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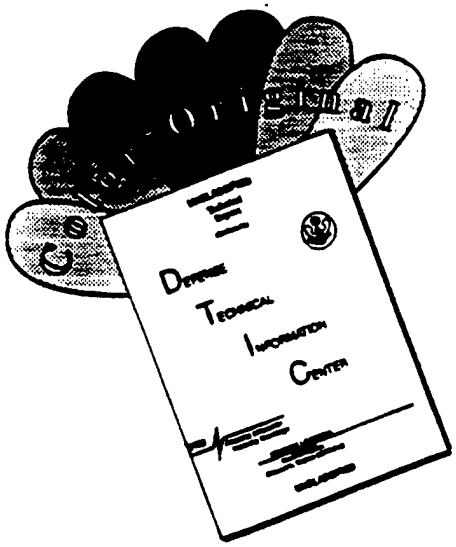

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Introduction:

General Background:

Breast cancer, like other epithelial tumors, is a highly complex and multi-factorial process. The biological events that occur and the causes are wide ranging. Among these events, genetic alterations, both somatic and inherited, are likely to play a major role. This premise has led to a broad search of both specific genes and chromosomal regions in efforts to correlate genetic changes to tumor behavior with the goal of improving diagnostic and therapeutic tools.

Background of previous work:

Human cancers are generally thought to arise through a multi-stage evolutionary process driven by inherited and somatic mutations of genes and clonal selection of variant progeny with progressively increasing tendency toward aggressive, unregulated growth. Progression occurs largely though somatic mutation of oncogenes and tumor suppressor genes, although some mutations in these same genes are inherited, making a given individual more susceptible to developing malignancy.

The range of oncogenes, tumor suppressor genes and chromosomal locations implicated in breast cancer is broad and ever expanding. These will not be discussed here, rather we will concentrate on the DCC and E-cadherin genes and their chromosomal locations, the focus of this grant.

Both DCC and E-cadherin have been implicated as tumor suppressor genes. The phenotype of a tumor suppressor gene is tumor progression as a result of alteration or loss of expression of the normal protein encoded by these genes. Often the phenotype is expressed as a result of loss of one of the two alleles present in normal cells. This allelic loss has been termed loss of heterozygosity (LOH). LOH has been postulated to inactivate the tumor suppressor gene located in the affected chromosomal region (Knudson's Hypothesis)(Knudson,1993). A region on chromosome 18q (18q21-23) has observed to show LOH in 35-70% of breast cancers(Thompson *et al.*,1993). This region contains the DCC gene. In colorectal cancers, DCC represents a classic tumor suppressor gene, showing LOH in over 70% of cases and grossly detectable somatic gene rearrangements in 15% of the cases (Cho and Fearon,1995).

E-cadherin maps to human chromosome 16q21.1 (Berx *et al.*,1995b), a region that has also been examined for LOH. Between 30 and 50% of breast cancer cases have shown LOH on 16q(Sato *et al.*,1991). Initially this was thought to correlate with decreased protein expression as multiple publications that showed decreased expression of E-cadherin in breast cancer(Gamallo *et al.*,1993; Moll *et al.*,1993; Oka *et al.*,1993; Rimm *et al.*,1995b; Siitonen *et al.*,1996), but more recently, only lobular carcinoma seems show E-cadherin mutations (Berx *et al.*,1995a). The significance of LOH at 16q remains to be shown. It is possible other tumor suppressor genes lie within that region.

Functionally, E-cadherin is well characterized. A hypothetical cartoon of the cadherin-based transmembrane adhesion complex (figure 1) shows the components of the complex and the associated cortical cytoskeleton. E-cadherin is shown as a dimer on the basis of recent structural studies on N-cadherin that showed it functions in a dimeric form (Shapiro *et al.*,1995). The Greek letters, α and β indicate the corresponding catenins. Plakoglobin has been shown to be identical to γ-catenin and is indicated by a γ. Although we have

evidence that α-catenin can bind both spectrin (Lombardo *et al.*,1994) and actin (Rimm *et al.*,1995a) and that it exists as a dimer (Koslov and Rimm, in preparation), the linkages shown are hypothetical. It is possible that it not only links the cadherin complex to the cytoskeleton by also participates in actin bundling in other parts of the cell or in other cell types. The question marks indicate that it is not known if catenin/spectrin and catenin/actin interactions can occur simultaneously. There is no evidence for direct interaction between E-cadherin and spectrin, but they co-localize and are suspected to be connected by either α-catenin or some yet unknown protein. The interactions between β- and α-catenin are shown as an example of β-catenin (a member of the *Arm* family) that binds directly to both E-cadherin and α-catenin. It is not yet known exactly which other members of the *arm* family can participate in this interaction. The stoichiometry of the interactions of β-catenins and plakoglobin are unknown although there is some evidence for a single *Arm* family member per cadherin. Plakoglobin, may participate, but probably not simultaneously with β-catenin. P120cas, the src substrate, may bind at a different site than the other members of the *arm* family, although there is also evidence that it binds at the same site. Protein tyrosine phosphatase μ is shown to indicate a direct connection to E-cadherin in some cells.

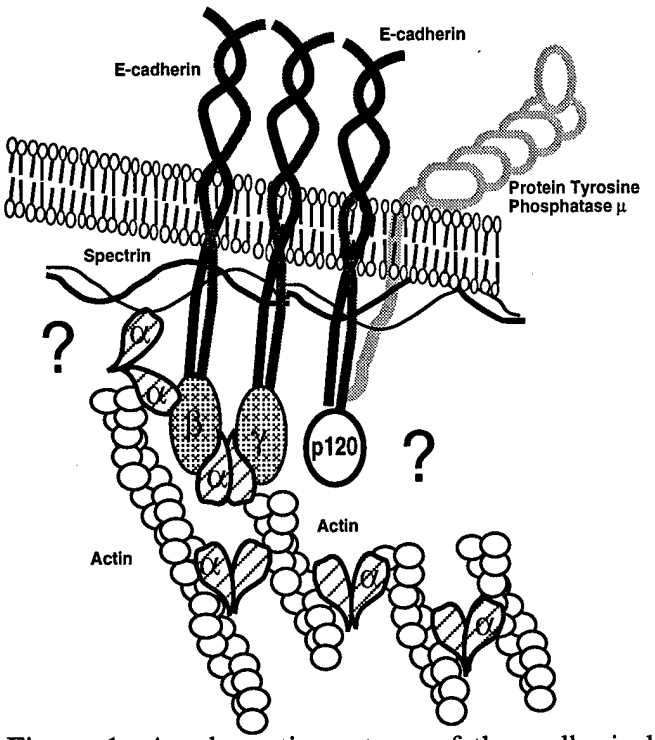


Figure 1 A schematic cartoon of the cadherin-based adhesive junction showing the proteins that interact with E-cadherin on the cytoplasmic face and their potential connections to the cytoskeleton.

Subject and Scope of Present Work:

In the original application, three technical objectives were proposed with the goal of understanding the role of the adhesion molecules DCC and E-cadherin in breast cancer. The initial task focused on use of LOH to assist in confirming that these adhesion molecules are important in the pathogenesis of breast cancer. Ultimately, the long term goals were to apply the findings of these studies to improving the diagnosis and management of breast cancer.

The following specific aims/tasks were proposed in effort to meet the above goals:

1) To determine the frequency of LOH affecting chromosomes 16q and 18q in primary breast cancers; to identify the common region affected by LOH on each of these chromosomes; and to identify the possible associations of such LOH events with clinical and histopathological features.

2) To identify specific genetic alterations in the DCC and E-cadherin genes in breast cancers

3) To address the functional role of the DCC and E-cadherin genes as tumor suppressor genes in breast cancer

Evolution of original aims due to progress in the field:

The original aims addressed above are still largely the direction of this effort. As there has been good progress in the field on many aspects of this work, as described both above and below, there has been some modifications in our focus. Primarily, less emphasis has been placed on Task 1, as copious evidence has developed that both DCC and E-cadherin are important in the pathogenesis of breast cancer. As the ultimate goal is to improve diagnosis and management of breast cancer, we have expanded our effort on two fronts. We have increased our efforts on the proteins associated to the cytoplasmic domain of E-cadherin, with emphasis on understanding both their function and regulation. We have also increased our efforts on understanding the regulation of expression of DCC and E-cadherin. As a result of our own work, as well as many others in the field, it is evident that mutation in these genes may represent an important, but less prominent mechanism of loss of function than alterations in the regulatory process.

Body:

SOW task 1: To determine the frequency of loss of heterozygosity (LOH) affecting chromosomes 16q and 18q in primary breast cancers; to identify the common region affected by LOH on these chromosomes; and to identify the possible associations of such LOH events with clinical and histopathological features.

Previous studies have provided evidence that LOH events affecting chromosomes 16q and 18q can be seen in a subset of breast cancers, and that the common region of LOH on 16q appears to include the E-cadherin gene and the common region of LOH on 18q includes the DCC (deleted in colorectal cancer) gene. Additional studies of LOH may serve to more precisely determine the frequency of LOH for each of these chromosomes, as well as to further localize the chromosomal region affected. In addition, the relationship of the LOH events to clinical and histopathological features of the cancers may yield important new information for assessing prognosis. Nevertheless, we have continued to emphasize studies of the leading candidate tumor suppressor genes from these chromosomes - E-cadherin on 16q and *DCC* on chromosome 18q. It should be noted that a new tumor suppressor gene from chromosome 18q21.1, termed DPC4 (for deleted in pancreatic cancer 4) has been identified. Mutations in the *DPC4* gene have been found in 40-50% of pancreatic cancers. However, recent studies suggest that *DPC4* is infrequently inactivated in other tumors, including breast cancer (Hahn,1996; Kim,1996; Schutte,1996; Thiagalingam,1996). Thus, *DCC* remains the leading candidate for the suppressor gene inactivated by 18q allelic loss in colorectal, breast, and other cancers. Finally, although our studies have provided strong supporting evidence for their inactivation in breast cancers, should we fail to obtain compelling evidence for E-cadherin and *DCC* inactivation in breast cancers, we plan to embark on studies to define the prevalence and chromosome regions affected by 16q and 18q LOH in breast cancers.

This section has been delayed for reasons described above in the section entitled "evolution of original aims due to progress in the field".

SOW task 2a: Studies of DCC and E-cadherin gene and protein expression in breast cancer cell lines

This section was completed in Year 1. See reprint from Pierceall et al, 1995 in Oncogene, in the appendix. The DCC aspect of this task is also addressed a manuscript by Meyerhardt et al., submitted to Oncogene. A copy of the submitted manuscript is included in the appendix

In light of changes in the studies outlined in the "evolution" section above, task 2a has been extended into the second year, with extensive focus on the mechanisms of E-cadherin expression. This work describes efforts to understand the E-cadherin gene expression by characterization of 5' flanking sequences and insertion of these sequences into a reporter gene (luciferase) system. Contrary to previous published work, (Graff *et al.*,1995) we find that modulation of expression is a function of trans-acting factors as opposed to changes in methylation of the promoter sequence. This work is described in the following manuscript in preparation.

Abstract

Decreased expression of E-cadherin (E-cad), a calcium-dependent transmembrane protein involved in cell-cell interactions, has been seen in a wide variety of epithelial cancers. In a subset of cancers, somatic mutations in the *E-cad* gene have been identified. However, in the majority of tumors, the mechanisms underlying loss of

E-cad expression are not well understood. A previous study suggested that in breast and prostate cancer cell lines *E-cad* expression was silenced by methylation of its promoter sequences (Graff et al., Cancer Research 55:5195-5199, 1995). We have generated luciferase reporter gene constructs containing human *E-cad* 5' flanking sequences and have studied their transcriptional activity in eight human breast cancer cell lines. The luciferase activity of the unmethylated reporter gene constructs closely paralleled the transcriptional activity of the endogenous *E-cad* gene in the cell lines. Furthermore, we were unable to obtain substantial reactivation of *E-cad* expression in the *E-cad*-negative cell lines by treatment with 5-aza-2'-deoxycytidine. Our studies suggest, therefore, that defects in trans-acting pathways regulating *E-cad* expression and not hypermethylation of the gene's promoter elements are the primary cause of decreased *E-cad* expression in breast cancers.

Introduction

E-cadherin (E-cad) is a calcium-dependent transmembrane protein of approximately 120 kilodaltons (kDa) that regulates epithelial cell-cell interactions at specialized regions of the plasma membrane termed adherens junctions. The function of E-cad depends critically upon its ability to link to the submembrane cytoskeletal matrix through its interactions with other cellular proteins, such as α-, β-, and γ-catenin/plakoglobin. Alterations in the structure or expression of E-cad or the catenins have been found to promote aberrant cell-cell interactions, and several lines of evidence suggest that *E-cad* may function as an invasion or metastasis suppressor gene. Decreased or undetectable levels of E-cad expression have frequently been seen in many different epithelial cancers. In some cancers, loss of E-cad expression has been associated with the loss of differentiated features and/or increased propensity of the cells to invade and metastasize to distant sites. In addition, the restoration of E-cad expression following *E-cad* gene transfer has been shown to inhibit the invasive and metastatic properties of the cells in in vitro and animal model systems.

Although it has been well-documented that E-cad expression is frequently decreased or absent in human cancers, the mechanisms underlying its loss of expression are not well-understood. The *E-cad* gene is located on chromosome 16q in a region which is frequent affected by allelic loss in several cancer types. Somatic mutations in the *E-cad* gene have been identified in more than one-third of gastric cancers of diffuse subtype, about 5 to 10 percent of endometrial and ovarian cancers, and in about 10% of breast cancer cell lines. The mutations identified include missense, nonsense, and splice mutations, as well as deletions. Nevertheless, in the majority of tumors where E-cad expression is altered, little is known about mutational mechanisms accounting for its reduced or absent expression. A recent study by Graff et al. has suggested that E-cad expression is silenced in breast and prostate cancers by methylation of the *E-cad* gene regulatory sequences. We report here the results of our studies to address the mechanisms underlying the loss of E-cadherin expression in breast cancers. In contrast to the conclusions of Graff et al., our findings suggest that defects in trans-acting pathways regulating *E-cad* gene expression, not methylation of its 5' regulatory elements, are the primary mechanisms underlying loss of E-cad expression in breast cancers.

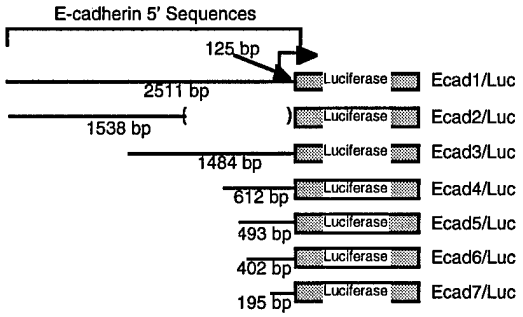
Materials and methods

Cell Culture. All cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in the recommended growth media. Cultures were incubated at 37°C with 5% CO_2, except for those maintained in

Lebovitz's L-15 media which were grown at 37°C without CO_2. Selected cell lines were treated with 5-aza-2'-deoxycytidine (Sigma Chemical Co., St. Louis, MO) at 1 μM or 3 μM for 5 days.

Plasmid constructs. Genomic clones containing human E-cad exons 1 and 2 as well as 5' flanking sequences were isolated from a human genomic DNA library generously provided by Jeremy Nathans using a human E-cadherin cDNA probe and multiple rounds of hybridization selection. An approximately 2.5 kb SalI-NcoI fragment extending 5' from the initiating methionine codon in exon 1 was identified and subcloned into pBluescriptII. Deletions of varying extent in the E-cadherin sequences were generated using exonuclease III and mung bean nuclease (Stratagene, La Jolla, CA). A series of luciferase reporter gene constructs containing E-cad 5' flanking sequences of varying extent was then generated by subcloning of the E-cad sequences into the SacI and HindIII sites of the pGL2-Basic vector (Promega, upstream of the coding region of the firefly luciferase

(Note, this manuscript is currently incomplete and will be included in its complete form in the 1997 annual progress report. The figures are shown with legends to allow interpretation of the data and the work is summarized in the conclusions section. (II))



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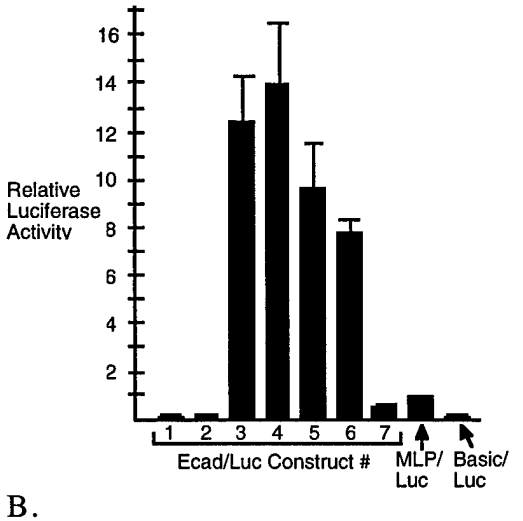

B.

Figure 2. Localization of promoter activity in human E-cadherin 5' flanking sequences. (A) For each luciferase reporter gene construct, the extent of sequences 5' to the E-cadherin initiating methionine is indicated. The proximal flanking

sequences deleted in the Ecad2/Luc construct are also indicated. The human E-cadherin transcriptional start site has been localized 125 bp upstream of the initiating methionine codon. In all reporter gene constructs, the E-cadherin initiating methionine codon has been destroyed, and a 28 bp flanking sequence separates the E-cadherin sequences from the initiating methionine of the luciferase gene. (B) Relative luciferase activity of the Ecad/Luc constructs and control constructs in MCF-7 breast cancer cells. Luciferase activities were determined by triplicate transfections of MCF-7 breast cancer cells with the indicated luciferase constructs and a pCMV-βGal control construct. All luciferase activities were normalized for β-galactosidase activity. The activity of an adenoviral late promoter luciferase (MLP/Luc) construct was assigned a value of 1 in each experiment, and the mean and standard deviation of the normalized luciferase activities are indicated for all other constructs.

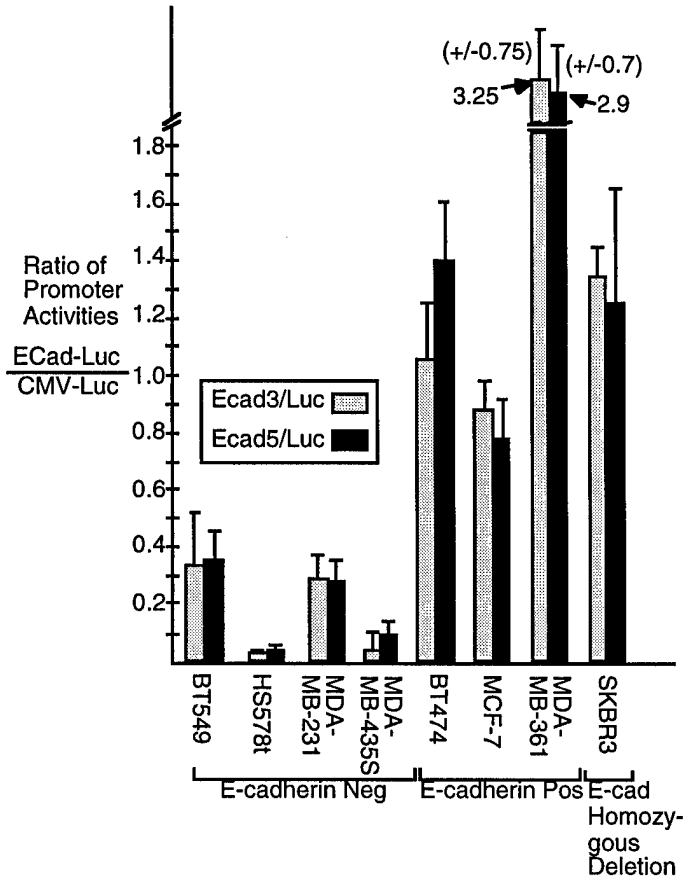

Figure 3. E-cadherin promoter activity is correlated with endogenous E-cadherin expression in breast cancer cell lines. The relative luciferase activity of two different E-cadherin luciferase constructs (Ecad3/Luc and Ecad5/Luc - see Figure 1) in eight breast cancer cell lines is indicated. Because transfection efficiencies varied among the cell lines, for each cell line, the luciferase activities of the two E-cadherin gene constructs were compared to the luciferase activity of a control CMV-driven reporter construct (pUHC-13-3). Luciferase activities in each cell line were determined by 3-5 independent experiments with the E-cadherin and CMV luciferase constructs and a pCMV-βGal control construct. All luciferase activities were normalized for β-galactosidase activity. The mean and standard deviation of the ratio of the luciferase activities are indicated. The E-cadherin expression status of the cell lines is shown.

Note that the SKBR3 cell line lacks E-cadherin expression because of a homozygous deletion in the E-cadherin gene.

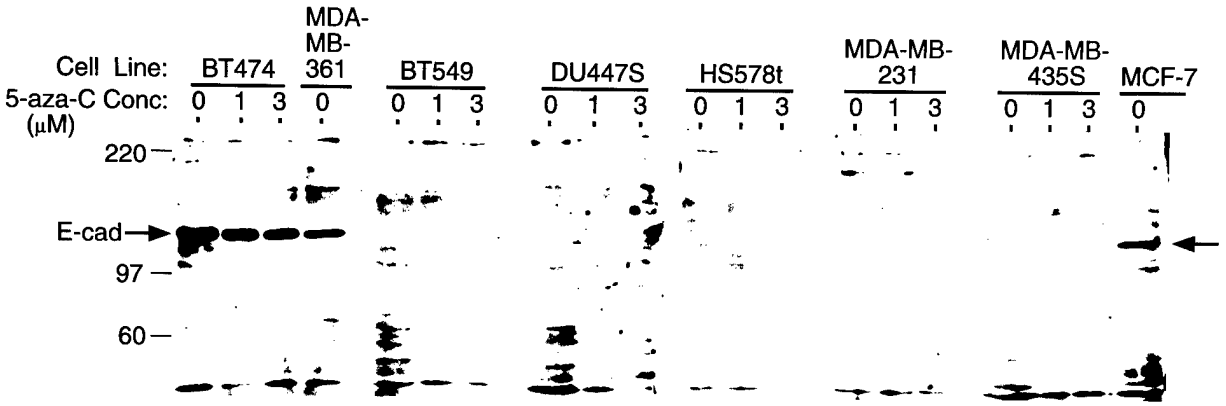

Figure 4. E-cadherin expression in breast cancer cell lines is not reactivated by treatment with 5-azacytidine. Immunoblot analysis was carried out to assess E-cadherin expression in cell lines following a five-day treatment with 5-azacytidine (5-aza-C) at 0, 1, or 3 μM. The identity of the cell lines is indicated at the top; the relative mobility of E-cadherin is indicated with an arrow; and molecular mass markers (in kDa) are indicated at the left.

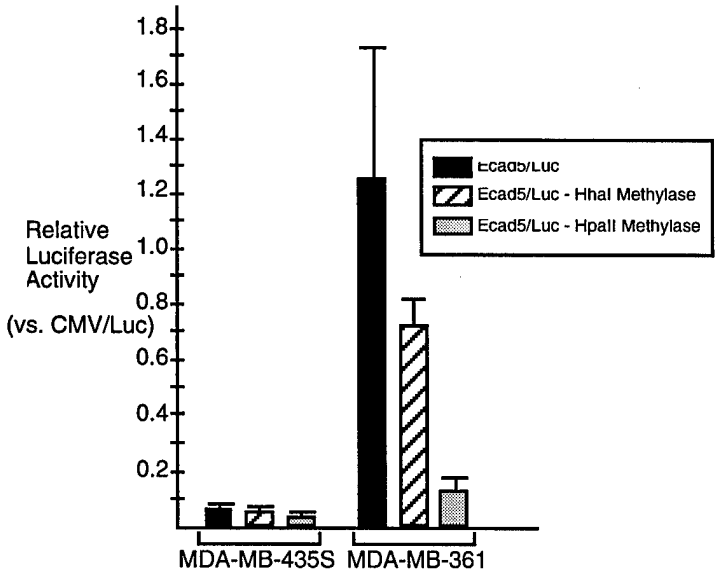

Figure 5. Methylation of the E-cadherin promoter can inhibit its activity. The relative luciferase activity of the E-cad5/Luc reporter gene construct was assessed in the MDA-MB-435S and MDA-MB-361 cell lines following in vitro methylation with either HhaI or HpaII methylase. Because transfection efficiencies differed between the lines, the luciferase activities generated by the Ecad5/Luc construct were compared to the luciferase activity of a control CMV-luciferase reporter construct (pUHC-13-3). Luciferase activities were determined by 3 independent experiments,

and all luciferase activities were normalized for β-galactosidase activity. The mean and standard deviation of the ratio of the luciferase activities are indicated.

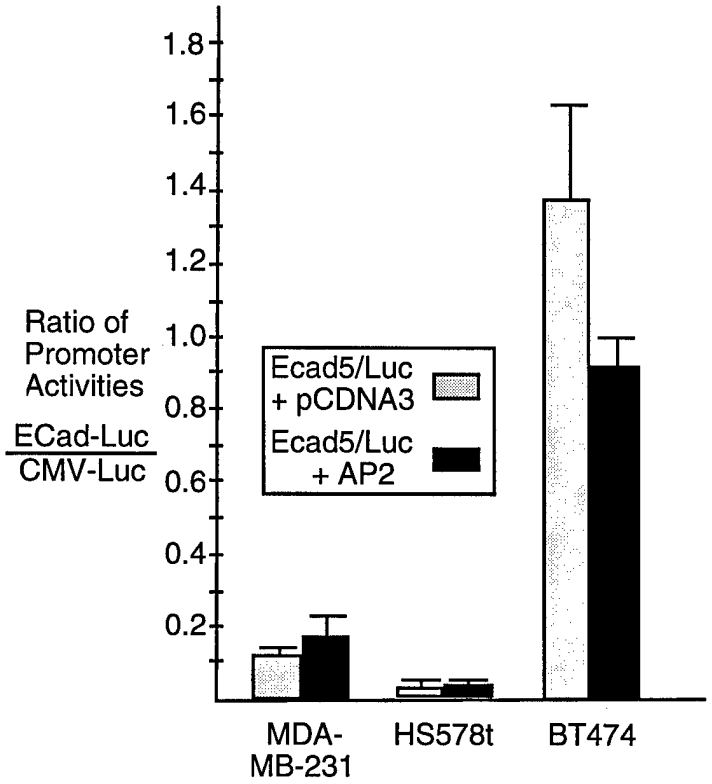

Figure 6. E-cadherin promoter activity is not activated by AP-2. Shown in the figure are the relative luciferase activities generated following co-transfection of three different breast cancer cell lines with the E-cad5/Luc reporter gene construct and either an expression vector encoding an AP2 cDNA or the empty expression vector. Because transfection efficiencies differed between the cell lines, the luciferase activities generated by the Ecad5/Luc construct were compared to the luciferase activity of a control CMV-luciferase reporter construct (pUHC-13-3). Luciferase activities were determined by 3 independent experiments, and all luciferase activities were normalized for β-galactosidase activity. The mean and standard deviation of the ratio of the luciferase activities are indicated.

SOW task 2b: Studies of DCC, E-cadherin and α- and β-catenin in primary tumors

Expression studies were undertaken primarily by immunohistochemistry as examinations based on mRNA will only be undertaken if protein expression alterations indicate a likely result by those methods. Immunohistochemical studies have focused on E-cadherin and cadherin associated proteins including α- and β-catenin and p120cas. This represents an extension of the original aims as discussed in the "evolution" section above. Studies of DCC on primary tumors, although desirable, have been hampered by lack of sufficient protein levels in either tumor or normal tissue for productive immunohistochemical studies.

The following section in from a manuscript in preparation related to cadherin and catenins expression in breast cancer:

ABSTRACT
BACKGROUND: Decreased expression of the homotypic cell adhesion protein E-cadherin has been reported in a variety of carcinomas, supporting a role for alteration in cell adhesion in invasion and metastasis. Functional cell adhesion requires a number of cytoplasmic cadherin-associated proteins, including α- and β-catenin. This study tests the hypothesis that altered localization of α-catenin and β-catenin in invasive breast carcinomas is associated with metastasis.
DESIGN: Using polyclonal antibodies to α-catenin and β-catenin we have examined staining patterns in routine formalin-fixed, paraffin-embedded sections from 47 infiltrating ductal breast carcinoma specimens, including 26 presenting with nodal disease. Cy3-conjugated secondary antibody was examined by immunofluorescence microscopy to allow subcellular localization. Results were compared with staining patterns observed using a monoclonal antibody to E-cadherin.
RESULTS: Normal breast epithelium reveals strong, linear, basolateral membranous adhesion protein staining at cell-cell borders. 14 of 21 (66%) of cases of infiltrating carcinoma in which axillary lymph nodes were negative for metastatic disease showed an altered membrane staining pattern. 22 of 26 (85%) of cases of infiltrating carcinomas with known nodal metastatic disease showed an altered membrane staining pattern.
CONCLUSION: These findings show that altered Ecadherin, α-catenin and β-catenin expression or localization is associated with nodal metastasis in breast cancer.

INTRODUCTION
The homotypic cell adhesion protein, E-cadherin, is important in the maintenance of the epithelial cell phenotype. In many cancers, reduced expression of E-cadherin has been associated with aggressive behavior and metastasis. Numerous are the studies showing an inverse relationship between levels of E-cadherin expression and severity or aggressiveness in many tumors including breast (Oka, et al.,1993), colon (Kinsella *et al.*,1993), prostate (Umbas *et al.*,1992), and gastric carcinoma (Mayer *et al.*,1993; Kawanishi *et al.*,1995), among others. Our own study (Rimm, et al.,1995b) showed reduction of α-catenin expression may be a more sensitive marker for cancer than E-cadherin.

A number of cytoplasmic cadherin-associated proteins, including α- and β-catenin link E-cadherin to the cytoskeleton. Proper anchorage to the cytoskeleton appears to be a requirement for E-cadherin function. Recent data has suggested that the catenins play a role in signal transduction, and that alteration of catenin expression is associated with aggressive tumors. Using fluorescent-conjugated antibodies we have conducted a retrospective study of 47 cases of ductal adenocarcinoma of the breast to test the hypothesis that altered α-catenin, β-catenin and E-cadherin expression or localization correlates with the presence of lymph node metastasis.

MATERIALS AND METHODS
Tissue Acquisition and Study Population: Formalin-fixed, paraffin-embedded invasive adenocarcinomas of the breast were obtained from archival material of the Pathology Department of Yale-New Haven Hospital. Equal numbers of lymph node-negative and lymph node-positive tumors were selected consecutively, in alphabetical order, from the eight-year period between 1982 and 1990. Patient information was obtained on all patients from the Connecticut Tumor Registry.

Antibody Preparation: Recombinant fusion proteins were prepared from full-length human cDNA clones for both α–catenin and β-catenin by expression in glutathione-S-transferase (GST)-based expression vectors (Pharmacia, Piscataway, NJ). Each

was purified on a glutathione affinity matrix and antisera were raised in rabbits by injection in complete Freund's adjuvant. Antibodies were affinity purified in two steps. Anti-GST activity was depleted by passage over a column of Affi-gel linked to GST. The eluent was subsequently passed over an Affi-gel column with bound α-catenin or β-catenin. After washing, antibodies were eluted with 100 mM glycine-HCl, pH2.5. Fractions containing active antibodies as detected by enzyme-linked immunosorbent assay (ELISA) were pooled, dialyzed into phosphate-buffered saline containing 1mM sodium azide and stored at -20. A commercial monoclonal antibody to E-cadherin was used (Transduction Laboratories, Lexington, KY).

Immunostaining: Paraffin sections were attached to glass slides, deparaffinized with xylene and 100% EtOH, and endogenous peroxidase activity was quenched with 0.5% hydrogen peroxide in methanol. Following hydration, slides were incubated in a pressure cooker 5-7 minutes(Norton *et al.*,1994), blocked with 0.3% BSA and incubated with primary antibody overnight. Slides were washed, incubated with Cy3-conjugated secondary anti-rabbit and anti-mouse antibody and stored in the dark at -20.

Scoring: Each slide was examined on at least two separate occasions by a pathologist and once by a technologist using a Zeiss Epifluorescence Microscope. The expression of each antigen was scored as Type 1, Type 2 or Type 3, according to the pattern demonstrated by at least 80% of the tumor cells. The Type 1 pattern was defined as continuous, linear basolateral staining at cell-cell borders, as seen in normal ductal epithelium. Tumors showing continuous linear staining with slight attenuation in width were included in this category. The Type 2 pattern was defined as non-continuous staining, usually granular or wavy, or mixed granular and wavy. The Type 3 pattern was characterized by segmental loss of staining over at least 50% of cell-cell membrane borders, accompanied by patchy irregular accumulations in intervening regions. (see Figure 7)

RESULTS and DISCUSSION

H & E, phase contrast and fluorescent immunostaining. All cases were examined by light microscopy and by phase contrast prior to examination by fluorescence microscopy, to verify the diagnosis of invasive ductal adenocarcinoma and to ascertain the presence of adequate tumor (at least three high-power fields) and an internal positive control (normal ductules and/or vessels).

α-catenin, β–catenin and E-cadherin immunostaining. Adjacent sections were stained with polyclonal antibodies to α- and β-catenin and a commercial monoclonal antibody to E-cadherin. Normal ductal epithelium showed continuous strong, linear basolateral membrane staining at cell-cell borders in contrast with the surrounding invasive tumor, which showed segmental loss of membrane staining. (Figure 8) Three staining patterns were distinguished. All cases are scored as Type 1 (normal pattern), Type 2 (discontinuous) or Type 3 (segmental loss), according to the pattern of membrane staining present in at least 80% of tumor cells. (Figure 7)

Distribution of cases by staining pattern. Forty-seven cases of primary breast carcinoma were evaluated for E-cadherin, α-catenin and β–catenin immunoreactivity. 17-19% of cases displayed the Type 1 (normal) staining pattern, 52-56% showed the Type 2 (discontinuous) pattern and 27-29% the Type 3 (segmental loss) pattern. High concordance of staining patterns with the three antibodies was found in 43 of 47 cases. (Figure 9)

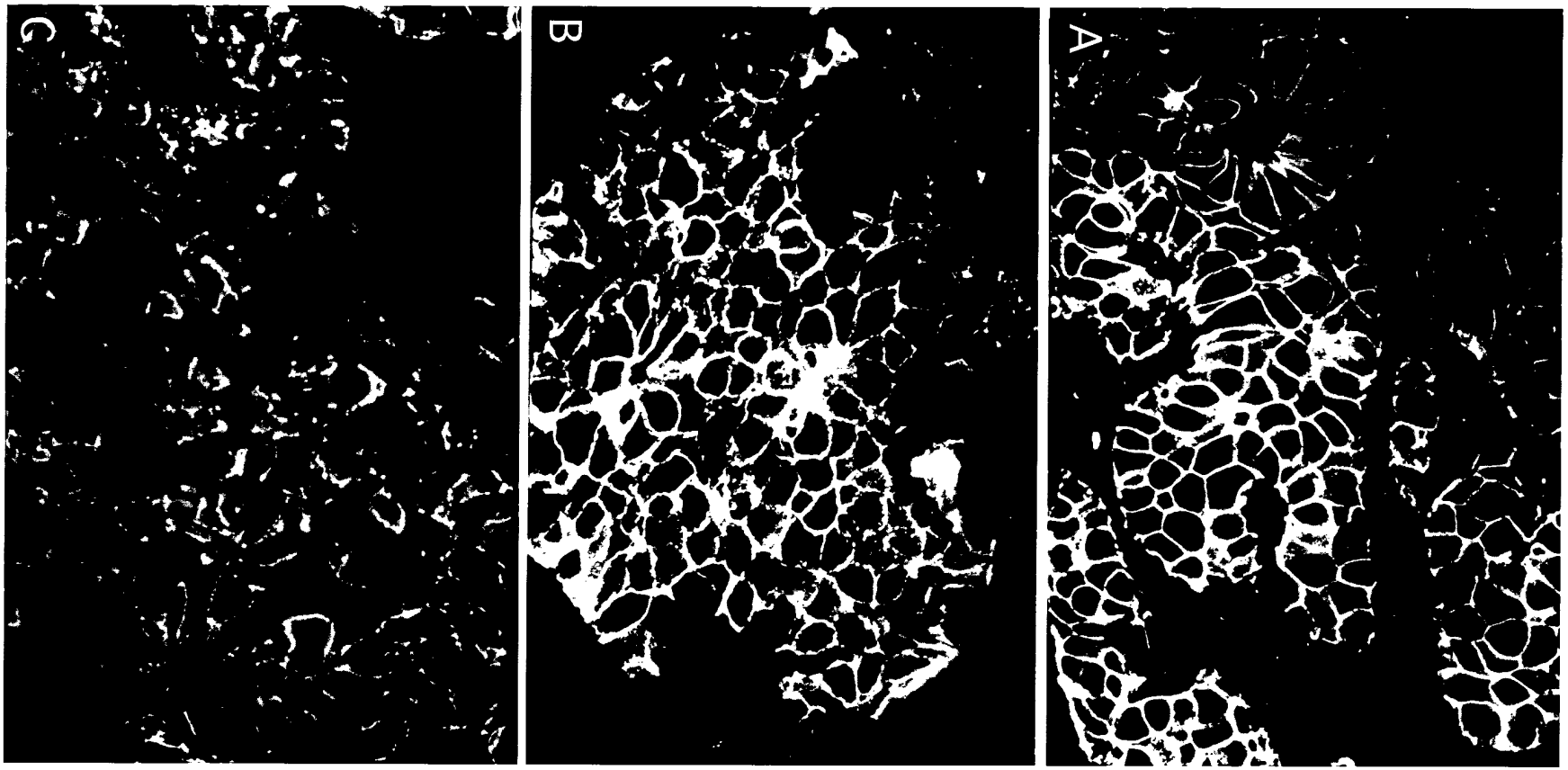

Figure 7. Staining of different cases with α-catenin to illustrate the three staining patterns used to score each case. Type 1 is illustrated by staining of tumor with a pattern indistinguishable from that seen in normal tissue (A). Type 2 pattern (B) shows a pattern we have called granular or discontinous, where staining is present but is not smooth and regular. Type 3 is a pattern of irregular staining that shows drop-out area or segmental loss of staining (C).

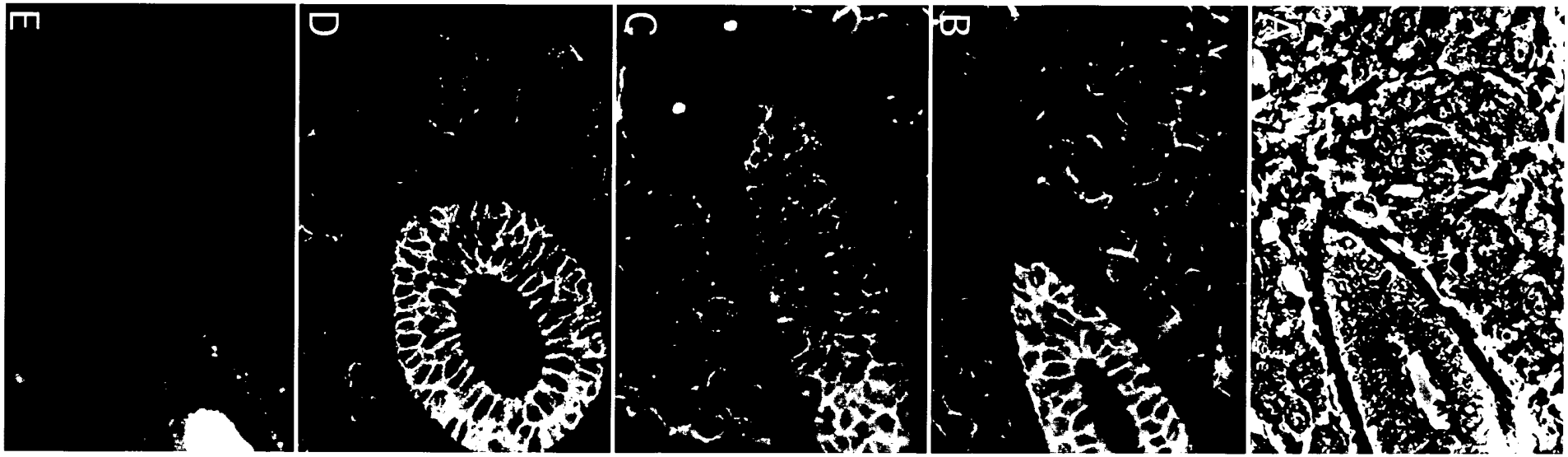

Figure 8. This figure illustates the normal staining patterns of the antibodies used for this study including a phase image of the tumor surrounding a normal duct (A), and staining with α-catenin (B), E-cadherin (C), β-catenin (D), and a rabbit preimune sera (E). Note the normal staining pattern in the duct, seen as the figure shows progressive sections through an individal normal duct surrounded by poorly differentiated tumor.

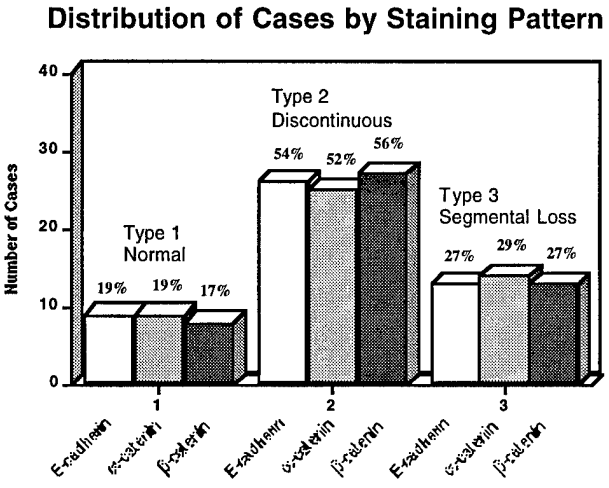

Figure 9. Distribution of staining pattern for each protein by staining pattern reflects the high concordance of staining seen in nearly all cases. Note also that 80+% of the cases had staining patterns that were abnormal.

Correlation of lymph node status with pattern of expression.
Cases with positive lymph nodes at presentation show increasing abnormalities in membrane staining for all three antigens. Those with negative lymph nodes demonstrate the opposite, with decreasing tendency for abnormal membrane staining. (Figure 10)

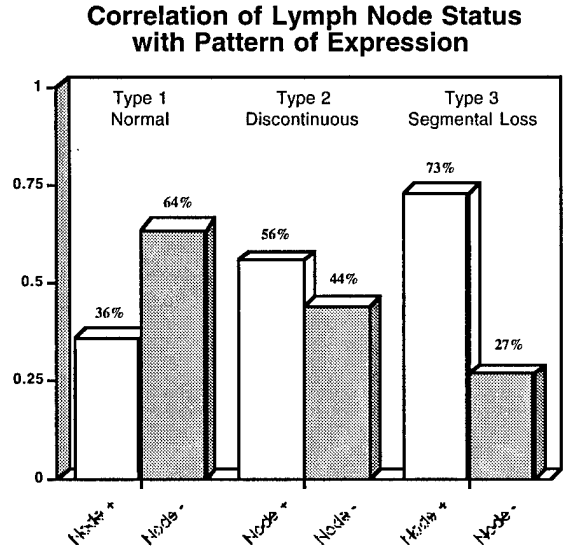

Figure 10 Distribution of staining patterns by node status at presentation. Node+ indicates that the patient had at least one positive axillary node at the time of primary tumor resection. Staining pattern types are as described above.

Metastasis, as assessed by the presence of positive lymph nodes, is associated with the alteration of expression of molecules in the cell adhesion cascade. Unlike

previous data from studies of breast cancer cell lines, expression patterns showed high concordance for all three antigens in essentially all cases. Altered patterns of expression are present in 80% of invasive ductal carcinomas of the breast for all three antigens. This figure is about 20% higher than most other studies, possibly due to greater sensitivity of this assay. A progressive increase in altered membrane expression was found to correlate with nodal positivity.

(Note, this manuscript is currently incomplete and will be included in its complete form in the 1997 annual progress report. The figures are shown with legends to allow interpretation of the data and the work is summarized in the conclusions section. (III))

A similar effort was undertaken to examine alteration of protein expression of the catenin-related E-cadherin binding protein p120CAS. This is described in the manuscript in preparation below:

Abstract

Expression levels of E-cadherin and associated proteins have been shown to have altered expression in human tumors. Since these proteins play an important role in establishment and regulation of adhesion, these alterations are suspected to be associated with the earliest stages of metastasis. Although alterations have been seen for E-cadherin and α-catenin, the 120kD *src* substrate called $p120^{cas}$ (CAS) has not yet been examined. Like other *arm* family members, CAS binds the cytoplasmic domain of E-cadherin and appears to play a role in regulation of adhesion. This work describes the of use monoclonal antibodies to show alteration or loss of expression of CAS in 61 cases of infiltrating ductal carcinoma of the female breast. CAS is altered or lost in 68%-84% of the cases, depending on how each case is scored. Surprisingly, alteration or loss of expression is seen more frequently in patients presenting without lymph node metastasis than those with higher stage disease (83% of node negatives vs. 54% of node positives). We conclude that this is evidence for regulated release of adhesion, as opposed to mutations in the components of the adhesive mechanism. In the 30 cases of node negative breast cancer, only 4 died of the disease, and each of these had alteration or loss of CAS expression. Although not statistically significant, this data suggests a correlation between loss of CAS expression and decreased survival in node negative patients.

Introduction:

Many studies have been done examining levels of expression of adhesion associated markers with the goal of identifying an association with metastasis. Cell-cell adhesion is primarily mediated by E-cadherin (see (Takeichi,1995) for review) and its associated cytoplasmic proteins, the catenins (see (Cowin and Burke,1996) for review). E-cadherin is has shown altered expression patterns in both ductal and lobular carcinomas(Moll, et al.,1993) and E-cadherin gene mutations have been seen associated with lobular carcinoma (Kanai *et al.*,1994)(Candidus *et al.*,1996). Alterations in expression of α-catenin expression have also been seen in breast cancer (Rimm, et al.,1995b) and breast cell lines (Pierceall *et al.*,1995). Although no correlation to survival has yet been shown for α-catenin, recently, E-cadherin alterations have been shown to be associated with decreased disease free survival (Siitonen, et al.,1996).

One adhesion associated protein that has not previously been examined for alteration of expression in any human tissues is $p120^{CAS}$ (CAS). This protein was first discovered as a substrate for a chicken src tyrosine kinase (Reynolds *et al.*,1989),

that was phosphorylated as a function of the presence of epidermal growth factor and other ligand induced signaling pathways (Downing and Reynolds,1991). More recently, this molecule has been defined as a member of the cadherin-based cell-cell adhesion complex(Reynolds *et al.*,1994)(Shibamoto *et al.*,1995)(Staddon *et al.*,1995). Cloning of CAS identified a characteristic 42 amino acid repeat placing it in the *arm* family with other cadherin associated proteins β-catenin and plakoglobin (Reynolds *et al.*,1992). Further characterization has shown that it binds to E-cadherin, like plakoglobin and β-catenin, but does not bind to either α-catenin or the APC protein (Daniel and Reynolds,1995). Although its function is still unknown, it is thought to play a role in modulation of adhesion as association of the tyrosine phosphorylated CAS with E-cadherin is elevated in *ras* transformed breast epithelial cell lines (Kinch *et al.*,1995).

The role of CAS in modulation of adhesion suggests it may be a good target for assessment of metastatic potential of tumors, as down regulation of adhesion or alteration of adhesion related proteins is a primary event in metastasis(Behrens,1993; Takeichi,1993). Unlike other adhesion proteins where there is loss of expression in tumor cell lines(Sommers *et al.*,1994; Pierceall, et al.,1995), CAS shows heterogeneous expression patterns, with isoform variability but no evidence of complete loss of expression in the lines examined (Mo and Reynolds,1996).

This finding suggests that we might not see alterations in expression in human tumor sections or alterations seen may be a function of alternative splicing of CAS. Although some isoforms of CAS have been defined (Mo and Reynolds,1996), others exist that are still in the process of characterization (Frans Van Roy, personal communication). This isoform diversity is unique for cadherin-associated proteins of the adhesion complex as no alternative spliced forms have been identified for β-catenin or plakoglobin and only a single alternative spliceform has been found for α-catenin (Rimm *et al.*,1994). The isoform diversity of CAS suggests that monoclonal antibodies will be a better tool for assessment of expression in human tumor sections. In this study we describe the use of 2 monoclonal antibodies to examine a series of cases of invasive ductal carcinoma of the breast. Surprisingly, both antibodies show a subset of tumors that show alteration or loss of expression of this protein. Furthermore, this loss has a paradoxical relationship to nodal metastasis, where patients presenting with invasion but without nodal disease are most likely to have altered expression.

Materials and Methods

Monoclonal antibodies. A series of monoclonal antibodies to murine CAS were prepared by the A. Reynolds lab and are described elsewhere (Wu et al, Hybridoma, submitted) Figure 11 shows a Western blot of these monoclonals showing specific binding.

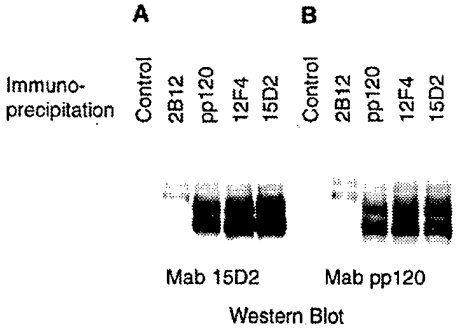

Figure 11. Specificity of p120-specific monoclonal antibodies. MDCK cell lysates were immunoprecipitated using the control Mab 12CA5 (Control) or the p120-specific Mabs listed across the top. Immunoprecipitates were separated on 8% polyacrylamide gels and then Western blotted with either Mab 15D2 (panel A) or Mab pp120 (B).

Patient population. This study is done a set of 60 patients who have had breast resections at Yale-New Haven Hospital between 5 and 15 years ago. All patients had invasive ductal breast cancer and approximately half of them presented with metastases to regional lymph nodes. The average age at diagnosis was 58.1. Average survival time is 5.6 years ranging from 39 days to 14.6 years. The 88% 10 year survival of node negative cases compared with the roughly 45% 10 year survival of node positive cases compares well with the literature and suggests that this is a representative population. This population is ethnically and racially diverse although, due to the nature of the disease, includes only females. All material was collected under the auspices of the Critical Technologies Program at Yale and in accordance with human investigation committee protocol #8219 to the principal investigator (D.L.R.).

Immunostaining. Standard histologic sections were cut from paraffin blocks and prepared for immunostaining using a pressure cooker antigen retrieval method(Norton, et al.,1994). Each section was baked at 60° C overnight, then deparaffinized and treated for antigen retrieval by immersion in 6.5 mM sodium citrate (pH=6.0) for 5 minutes in a conventional pressure cooker (KMart). Sections were then blocked with 3% BSA in Tris buffered saline (TBS) (150mM NaCl, 20mM Tris pH=8). Monoclonal antibodies were diluted to 7 ng/ml and incubated in a humidity chamber overnight before washing 7 times with TBS including 0.01% triton X-100 in the 6th wash. For increased sensitivity and better subcellular localization, Cy3 conjugated second antibodies were used instead of the conventional enzymatic reaction-based chromogens. Cy3-Goat anti-mouse antibody (Jackson ImmunoResearch Labs, West Grove, PA) was diluted 1:500 in TBS with 3% BSA and placed in the sections for one hour before washing as above and coverslipping. Slides are stored at -20°C to maintain the fluorescent signal, which appears to be stable for over one year using these conditions.

Histologic scoring and analysis. Cases were examined on an Olympus AX-70 epifluorescence photomicroscope by a technician (T.D.) and twice by a pathologist (D.L.R.) and scored as described below. In each case, a serial hematoxylin and eosin section was examined for orientation and confirmation of the histologic diagnosis. Each case was scored blindly with respect to patient history, presentation and previous scoring. Patient follow-up information was obtained from Dr. Diane Fisher in conjunction with the Yale Comprehensive Cancer Center and the Connecticut Tumor Registry. Data analysis was done using StatView4.5 for Macintosh.

Results

To assess the tissue and subcellular localization of CAS, tissue samples from a range of breast disease were examined. In normal tissue, as well as benign disease and all cases of ductal carcinoma in situ (DCIS) examined, the expression pattern is indistinguishable from the normal membrane staining pattern seen for E-cadherin, α-catenin and β-catenin.

In tumors, a range of expression was seen. Using monoclonal 12F4, some tumors gave a staining pattern that was strong, membrane localized, and although clearly in malignant cells, not distinguishable from the normal ductal cells (Figure 12A-B). Other cases showed a heterogeneous staining pattern with some cells showing staining in some regions where other regions showed reduced intensity of staining or complete absence of fluorescence (Figure 12C-D). In other cases there was complete loss of expression of this epitope (Figure 12E-F),but normal ducts stained within the same section stained brightly. This internal positive control was present in nearly all of the cases and allowed consistent interpretation of loss of expression.

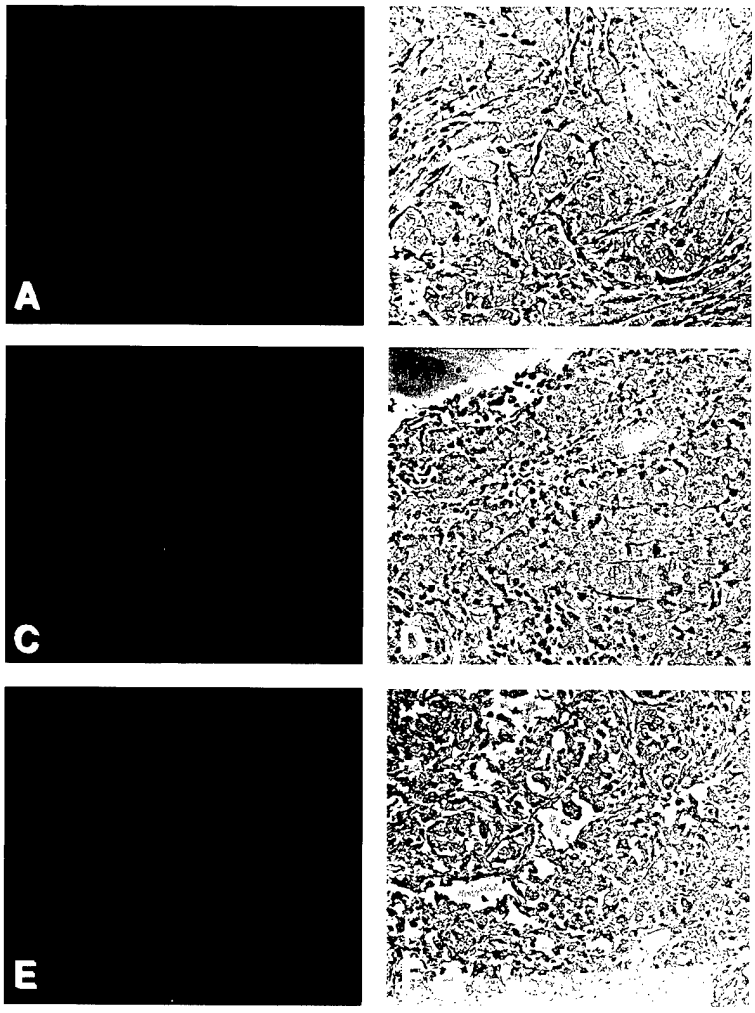

Figure 12. Examples of the 3 staining patterns used to score each breast cancer case with accompanying phase image of the tumor. "Normal" staining (A) is defined as cases will staining patterns in the tumor that are indistinguishable from normal regions of the same slide. "Altered" staining (C) is segmental loss or decreased intensity compared to normal. "Lost" is a pattern of near complete loss of staining (E) in both intensity and pattern. Corresponding phase images are also shown (B,D,F) to illustrate that no morphologic distinction was associated with the altered staining patterns.

To quantitate and correlate the staining patterns the cases were scored in 3 categories defined as follows: 1) Normal, indistinguishable from normal intensity in at least 90% of the cells; 2) Altered, heterogeneous staining with variable levels of intensity; and 3) Lost, complete absence of the normal staining pattern with less than 10% of cells within a given region showing even light heterogeneous staining. Examples of each of these patterns are illustrated in Figure 12. Each case was scored estimating an overall average score for the entire section and a "worst area" score for regions (at least the size of a 40X field) where staining intensity was the lowest. This system was used to reflect the fact that many of the cases showed regions of heterogeneous staining in some areas with complete loss in other areas or more rarely, predominantly normal staining with smaller regions of heterogeneous staining. Although many cases contained regions of DCIS, these areas were not included in the scoring of each case. Each case was examined twice to confirm category selection.

To confirm the staining pattern and address the isoform issue, a second monoclonal antibody (15D2) that appears to recognize all currently characterized isoforms was used. A random selection of about half (30) of the cases was scored separately with this antibody. In each case, the staining pattern was similar to that seen for 12F4, but generally cytoplasmic staining was slightly more prominent (data not shown). No case showed a significantly different staining pattern, such that it would be scored in a different category.

Scoring of the 61 cases in the study is summarized in table 1. As has been seen with other adhesion related proteins, there is loss or alteration of expression of CAS in at least 68%. If the subregion of the specimen that is most altered is more representative of the biology of the tumor, then as many as 84% of the cases show alteration in CAS expression.

	Average Pattern		Worst Pattern	
Category	count	percent	count	percent
normal	19	31%	10	16%
altered	21	34%	12	20%
lost	21	34%	39	64%
total	61		61	

Table 1. Summary of p120CAS staining pattern by category in 61 cases

To evaluate the clinical utility of this expression pattern correlations with other prognostic parameters were assessed. The most predictive parameter for survival in breast cancer patients is metastasis to lymph nodes, with node negative patients enjoying significantly better survival. Division of CAS expression on the basis of nodal status at presentation shows a paradoxical pattern (figure 13). Examination of averaged expression areas of node negative patients, that might be expected to retain expression, show a high frequency of loss, with a total of 83% of the population showing at least alteration of expression. Conversely, in node positive patients where alteration might be expected to be more common, 45% of the cases show expression indistinguishable from normal.

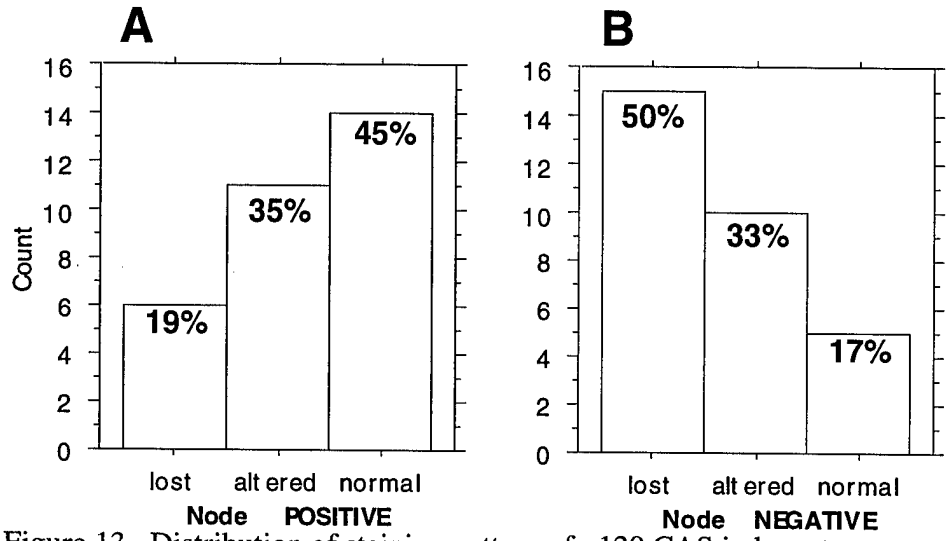

Figure 13. Distribution of staining pattern of p120 CAS in breast cancer patients divided by node status on presentation

Other parameters commonly assessed with prognostic value include hormone receptor status, patient age and tumor size. As these cases were predominantly done in the mid to late 80's, estrogen and progesterone receptor studies were not done on most of the cases. No relationship was found with patient age or tumor size.

Finally, we examined the predictive value of CAS with respect to long term survival. Again cases were split into those presenting with metastases to nodes and those without. Kaplin-Meier analysis was done on each group on the basic of the CAS expression pattern (figure 14). In both cases only trends can be assessed as the sample size is small in this initial study. In node positive cases there is both altered and lost CAS expression cases have a 8 year survival in the 40% range and while CAS normal patients show 8 year survival near 75% (figure 14A). Similarly in node negative cases there appears to be decreased survival associated with decreased CAS expression in that the group with lost expression shows 80% survival at 8 years

while none of the 5 node negative cases with normal CAS expression have died of the disease (100% 8 year survival)(figure 14B).

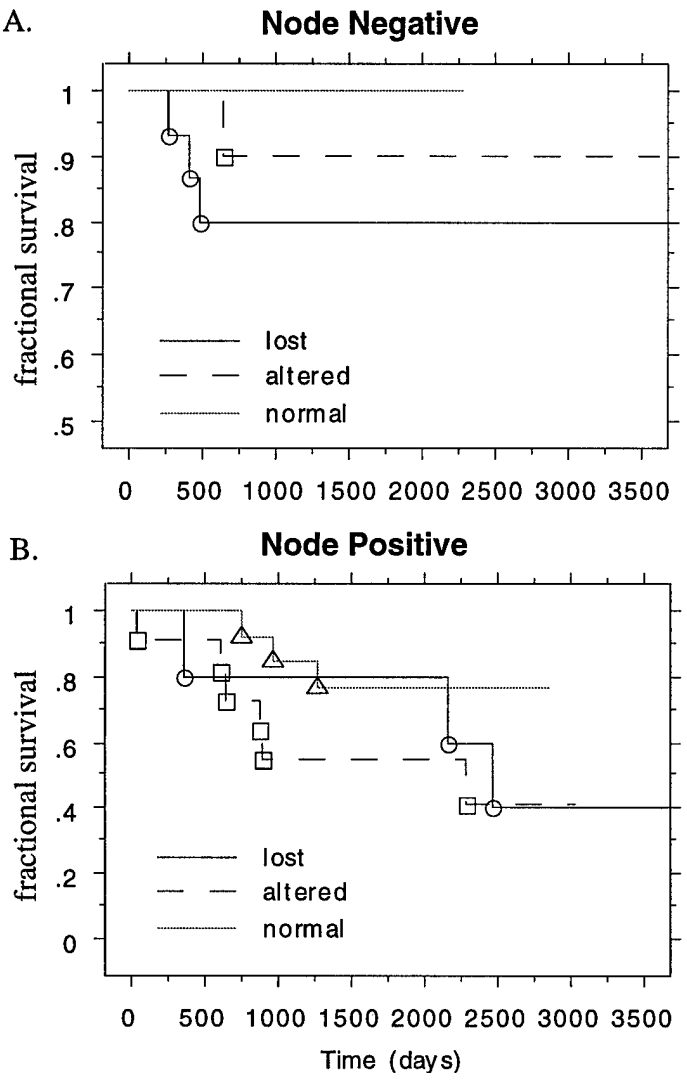

Figure 14. Kaplan Meier Analysis of survival of patients without (A) and with (B) lymph node metastasis at presentation, divided by the p120CAS staining pattern.

Discussion

A range of studies have found that E-cadherin(Gamallo, et al.,1993; Moll, et al.,1993; Oka, et al.,1993; Siitonen, et al.,1996), and more recently, α-catenin(Ochiai *et al.*,1994; Rimm, et al.,1995b) expression are altered in breast cancer. The consensus in these papers is that there is increased alteration or complete loss of expression of the adhesion proteins, and that increased alteration generally correlates to increased stage or grade of tumor. This work suggests that CAS is another example of an adhesion associated protein whose alteration is associated with worse outcome.

The CAS data is somewhat complicated by data from cell lines that show no examples of loss of expression (Mo and Reynolds,1996) and show expression of multiple isoforms. In the case of other cadherin associated adhesion molecules, at least one cell line can be found in which expression is altered. In some cases many lines show loss of expression (Pierceall, et al.,1995). It is also possible that cell lines examined to date, do not represent the stage in oncogenesis that shows alteration of expression in human tissue sections. Although 23 lines have been examined (Mo and Reynolds,1996), it is possible that rare lines will be found that show complete loss of CAS.

Examination of tumor cell lines also reveals that the isoform diversity of this molecule far exceeds that seen for any other molecules in the cadherin-based adhesion complex (Mo and Reynolds,1996). Although the complete isoform story is yet to be completed for human CAS, the monoclonal antibodies used for this study were selected since they recognize all known isoforms of this protein. If isoforms exist that are not recognized by these antibodies, it is possible that this study is detecting a dramatic isoform switch associated with invasion, and possible survival.

The association of loss of adhesion markers with lymph node metastasis is equivocal. The prediction that loss or alteration in expression would be correlated with lymph node metastasis is confirmed in some works(Oka, et al.,1993; Siitonen, et al.,1996), but not others(Gamallo, et al.,1993). Furthermore, we (Dillon *et al.*,1996) and other investigators(Shimoyama *et al.*,1989) have seen expression patterns of adhesion proteins that are indistinguishable from normal in tumors within lymph nodes. One conclusion of this finding is that alteration of expression may be a function of regulatory processes. This concept is amplified by this work since the correlation to lymph node metastasis is the opposite of that generally expected. That is, we see more dramatic alterations in expression in node negative cases than node positive cases. This is of particular interest, as there are fewer useful prognostic markers in node negative patients.

Survival is the ultimate outcome that any marker seeks to predict. In this case, there appears to be a trend toward decreased survival associated with decreased CAS expression. Although we had hoped to reach statistical significance, larger numbers will be required. The most interesting group are the node negative cases. About 58% of cases present in this manner and they have somewhere between 80 (Berg and Hutter,1995) and 97% 5 year survival (Parker *et al.*,1996). Given this survival rate, as few as 1, and at most 6 patients would be expected to die in our cohort of 30 node negative patients. In fact, we recorded 4 events within 5 years after presentation, 3 of whom had complete loss of expression and 1 that showed altered expression. Since no patients in the "normal expression" group died of disease during the study period, it is not possible to test for significance (at least using the Mantel Cox Log Rank test). In spite of the lack of significance due to our small numbers, this study suggests that alteration of CAS expression in node negative patients may predict outcome. Further, larger studies of node negative patients are underway.

SOW task 2c.1: Studies to identify and characterize specific mutations in E-cadherin genes

Specific efforts to find mutations in E-cadherin in human breast tumors has met with limited success elsewhere in the field. Although lobular carcinoma of the breast has been found to have some gene mutations by three groups (Kanai, et al.,1994; Berx, et al.,1995a; Candidus, et al.,1996), no mutations in E-cadherin have yet been published for any cases of ductal carcinoma. Examination of tumors for specific mutations of E-cadherin

may be done in years 3 and 4, with some modification of the original methods to reflect the successful techniques of Berx and colleagues (Berx, et al.,1995a).

SOW task 2c.2: Alterations in *DCC* expression and gene structure in breast cancers

We have assessed DCC gene expression in a panel of breast cancer cell lines using ribonuclease (RNase) protection and reverse transcription polymerase chain reaction (RT-PCR) assays. DCC expression is not detectable by RNase protection in the cell lines studied thus far nor is it detectable by RT-PCR in the majority of cell lines studied (Meyerhardt et al. manuscript and unpubl. observ.). We have recently developed protocols for the detection of DCC expression by immunohistochemical approaches (Reale,1996) and we will use this approach to assess DCC protein expression in primary breast cancers. We have undertaken Southern blot analysis of a limited number of breast cancer cell lines using DCC cDNA probes and no alterations were found (unpubl. observ.). Because the vast majority of breast cancer cell lines lack DCC transcripts and the *DCC* exons are distributed over greater than 1.35 million basepairs at chromosome 18q21.2, we have not undertaken a sequence-based analysis of the *DCC* gene structure.

SOW task 3a: Transfection, isolation and preliminary characterization of breast cancer lines with E-cadherin, β- and α-catenin and DCC cDNAs.

Efforts toward this task are well underway but have met with limited success. We have produced cDNAs encoding all 4 proteins and successfully cloned them into a range of eukaryotic expression vectors. We have focused predominantly on the CMV driven systems and either lipofection or electroporation as mechanisms for transfection. To date we have been unable to produce and stable transformants of α-catenin or E-cadherin in any breast cancer cell lines. We have had limited success in a non-breast cancer line (Clone A) and are using this system to optimize conditions. Unfortunately, the transformation of these genes seems to confer a tremendous (and as yet insurmountable) growth rate disadvantage, and although we are able to see transient expression and even stable transfection by immunofluorescence at early time points, minimal passaging of the cells to produce sufficient numbers to analyze or even freeze have resulted in loss of expression.

Similar difficulties have been encountered with DCC transfection. We have undertaken transfection of two breast cancer cell lines that lack endogenous DCC expression - SK-BR-3 and BR474. As we proposed in our initial application, we have utilized three different CMV-driven mammalian vectors: pCMV/DCC-S, a vector encoding full-length DCC; pCMV/DCC-T, a vector encoding a DCC form lacking the majority of its cytoplasmic domain sequences; and pCMV/DCC-M, a control vector encoding a severely truncated form of DCC. Thus far, we have picked more than 40 G418-resistant clones following transfection of the SK-BR-3 cell line with each of the three vectors (Table 2). These clones have been expanded and Western blot analysis analyses have been carried out to identify clonal lines expressing full-length or cytoplasmic-truncated forms of DCC. None of the 15 cell lines transfected with the pCMV/DCC-S vector and analyzed by Western blot analysis have been found to express DCC. Only two of the 18 lines transfected with the pCMV/DCC-T vector stably express the truncated protein.

Table 2. Transfection of SK-BR-3 with DCC constructs

	# of Independent Colonies Reaching Indicated Point of Analysis			
Construct	24-well	T-25 Flask	Western Blot	# of DCC-Pos*
pCMV/DCC-S	43	16	15	0
pCMV/DCC-T	54	20	18	2
pCMV/DCC-M	47	17	4	0

* - DCC protein expression; note no DCC protein expression is predicted from the DCC-M construct because the predicted mutant protein would be truncated immediately downstream of the signal sequence at codon 57.

Thus far, only a relatively limited number of G418-resistant clones from transfections of the BT474 cell line have been analyzed for DCC expression, and no stably expressing clones have been obtained thus far (data shown). Because we and others have previously used the CMV-driven DCC expression vectors to generate a number of mammalian cell lines with stable expression of full-length and truncated forms of DCC (Pierceall,1994)(Klingelhutz,1995) (E.R. Fearon, unpublished observations), our findings, though preliminary, suggest that DCC expression may cause growth arrest or promote cell death in transfected breast cancer cell lines. To better address this hypothesis, we have generated constructs in which DCC cDNAs have been placed downstream of tetracycline-responsive elements (Gossen,1992). Using these constructs, we hope to generate breast cancer cell lines in which DCC expression can be tightly regulated by the levels of tetracyline in the culture media. Our preliminary studies suggest that inducible expression of DCC can be obtained following transfection with these vectors, although the vectors appear to confer somewhat leaky constitutive expression of DCC in the presence of tetracycline in several human cancer cell lines. Finally, we are in the process of generating recombinant adenoviral constructs containing the various DCC cDNAs. Because of the high efficiencies of gene transfer obtainable with adenoviral vectors, these vectors should be particularly useful for rapid assessment of the effects of DCC expression on breast cancer cell growth.

SOW task 3a: Further characterization of the *in vitro* growth properties of breast cancer lines transformed with E-cadherin, β- and α-catenin and DCC cDNAs.

Although this task is targeted for years 2-4, it is dependent on the success of task 3a, which has not yet occurred.

Conclusions:

I. Expression of E-cadherin, α-catenin and β-catenin in breast cancer cell lines is altered in a large number of cases.

Alterations are often, but not always, seen in one member of the complex where the others are expressed normally.

Alterations occur at both the protein and message levels in breast cancer cell lines.

II. Using a luciferase reporter gene, the E-cadherin promoter shows transcriptional activity in 3 breast cancer lines that express E-cadherin protein as well as 3 lines that do not.

Expression of the reporter gene in an unmethylated construct parallels that of the endogenous E-cad gene in each cell line, suggesting methylation does not down regulate the E-cad promoter Furthermore, treatment with 5-aza-2'deoxycytidine does not reactivate E-cad in lines where E-cad is not transcribed. We conclude the down regulation of transcription at the E-cad promoter is not a function of methylation but rather a trans-acting factor.

III. Expression patterns showed high concordance for all three antigens in essentially all human breast cancer cases, unlike previous data from breast cancer cell lines.

Altered patterns of expression are present in 80% of invasive ductal carcinomas of the breast for all three antigens.

A progressive increase in altered membrane expression correlates with nodal positivity.

Metastasis, as assessed by the presence of positive lymph nodes, is associated with the alteration of expression of molecules in the cell adhesion cascade.

High levels of concordance of expression between all three proteins and rare examples of complete loss of expression, along with the fact that 36% of cases with normal α-catenin expression are node positive (have metastasized) suggesting that down regulation of the adhesion molecules (at either the transcriptional or translational level) must play a more significant role than somatic mutation in affecting loss of adhesive function.

IV. Expression of p120CAS shows more dramatic alteration than other catenins, with frequent complete loss of expression, even using sensitive immunofluorescence techniques.

As many as 64% of cases showed at least some area of tumor with complete loss of expression and as many as 84% showed some alteration in expression.

The correlation of p120CAS expression with lymph node metastasis is the opposite of that expected. That is, 83% of node negative cases showed some alteration while 45% of node positive cases showed staining patterns indistinguishable from normal. Although our numbers in this study are small, the loss of expression appears to be predictive of poor outcome in node negative cases.

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Abstracts
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Appendix:

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Frequent alterations in E-cadherin and α- and β-catenin expression in human breast cancer cell lines

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Alterations in intercellular junction and membrane cytoskeletal proteins may underlie some of the morphological, invasive and metastatic properties of cancer. E-cadherin, a transmembrane protein that functions in epithelial cell-cell interactions at adherens junctions, is linked to the membrane cytoskeletal matrix through interactions with α- and β-catenin. We have carried out studies of *E-cadherin* and α- and β-*catenin* in 18 breast cancer cell lines to determine the prevalence and nature of alterations in these genes in breast cancer. Ten lines failed to express E-cadherin protein at detectable levels and seven failed to produce detectable levels of *E-cadherin* transcripts. In a line lacking E-cadherin expression (SK-BR-3) a homozygous deletion of a large portion of the *E-cadherin* gene was noted. Localized sequence alterations in *E-cadherin* transcripts were not identified in any lines. In contrast to the results of a previous study, no relationship was identified between *E-cadherin* expression and HER-2/NEU expression. Two of the 18 lines had no detectable α-catenin protein and six others had reduced levels. The two lines lacking α-catenin protein had reduced or undetectable levels of α-*catenin* transcripts, while no consistent changes in α-*catenin* transcript levels were seen in the lines with reduced, but detectable, levels of α-catenin protein. Similarly, although two lines lacked β-catenin protein, in most lines the levels of β-*catenin* transcripts and protein were not well correlated with one another. Our findings suggest that alterations in E-cadherin and α- and β-catenin expression are frequent in human breast cancer-derived cell lines, and that in some cases the decreased expression may result from mutations in the genes. Furthermore, the frequent alterations in the expression of these proteins argue that loss of function in the E-cadherin-catenin pathway may be critical in the development of many breast cancers.

Keywords: breast cancer; E-cadherin; α-catenin; β-catenin; cytoskeleton; cell-cell interactions

Introduction

The cadherins are a family of Ca^{2+}-dependent transmembrane proteins which appear to mediate cell-cell interactions through homotypic interactions (Takeichi, 1991; Tsukita *et al.*, 1992). E-cadherin is expressed on epithelial cells and its function depends upon the ability of its cytoplasmic sequences to link to the submembrane cytoskeletal matrix through interactions with proteins termed the catenins (Nagafuchi and Takeichi, 1988; Ozawa *et al.*, 1989; 1990; Kintner, 1992). The catenins include α-catenin, a protein with similarity to the actin-binding protein vinculin (Nagafuchi *et al.*, 1991; Herrenknecht *et al.*, 1991); β-catenin, a relative of the *Drosophila* armadillo protein which functions in the determination of segment polarity (McCrea *et al.*, 1991; Pfiefer and Wiechaus, 1990; Butz *et al.*, 1992) and γ-catenin, which is identical to plakoglobin and is found in both adherens junctions and desmosomal junctions (Francke *et al.*, 1989; Knudsen and Wheelock, 1992; Hinck *et al.*, 1994; Nathke *et al.*, 1994). Functional interactions between epithelial cells may be abrogated not only by defects in E-cadherin structure or expression, but also by alterations in catenin expression or structure (Hirano *et al.*, 1992).

Decreased or undectable levels of E-cadherin expression have been noted in many immunohistochemical studies of epithelial cancers (Schipper *et al.*, 1991; Shimoyama and Hirohashi, 1991a,b; Shiozaki *et al.*, 1991; Umbas *et al.*, 1992; Brabant *et al.*, 1993; Bringuier *et al.*, 1993; Doki *et al.*, 1993; Dorudi *et al.*, 1993; Gamallo *et al.*, 1993; Mayer *et al.*, 1993; Moll *et al.*, 1993; Morton *et al.*, 1993; Oka *et al.*, 1993; Rasbridge *et al.*, 1993; Kadowaki *et al.*, 1994; Rimm *et al.*, 1995). In some tumor types, the loss of E-cadherin expression has been associated with loss of differentiated features in the tumor. In addition, the loss of E-cadherin expression in some cancers has been found to correlate with an increased likelihood of distant metastasis in the patient, suggesting a potential role for E-*cadherin* as an invasion or metastasis suppressor gene. More direct experimental support for this proposal has been obtained from in vitro studies of several rodent and human tumor cell lines in which loss of E-cadherin function is correlated with the acquisition of invasive properties (Behrens *et al.*, 1989; Frixen *et al.*, 1991; Vleminckx *et al.*, 1991; Birchmeier *et al.*, 1993).

Further evidence that loss of E-cadherin function may be critical to tumorigenesis has been provided by other studies. The chromosome 16q region containing the E-*cadherin* gene is affected by loss of heterozygosity (LOH) in breast and prostate cancers (Sato *et al.*, 1990; Bergerheim *et al.*, 1991; Carter *et al.*, 1991; Lindblom *et al.*, 1993). Somatic mutations in the E-*cadherin* gene have been identified in some gastric carcinomas, particularly diffuse type gastric cancers (Becker *et al.*, 1994; Oda *et al.*, 1994). In addition, a small subset of 130 endometrial and ovarian tumors studied were found to have somatic missense and nonsense mutations in the E-*cadherin* coding region (Risinger *et al.*, 1994). Finally, in some breast cancer lines and a

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prostate cancer cell line, evidence has been obtained that altered transcriptional regulation may account for loss of E-*cadherin* expression (Behrens *et al.*, 1991; Bussemakers *et al.*, 1994). Nevertheless, in the majority of cancers where altered E-*cadherin* expression has been observed in immunohistochemical studies, the mechanisms underlying its altered expression remain poorly understood.

Alterations in the catenins have also been seen in some human cancers, Decreased or absent α-catenin expression has been noted in some primary breast, esophageal, and prostate cancers (Shimoyama *et al.*, 1992; Morton *et al.*, 1993; Kadowski *et al.*, 1994). Genetic alterations at the α-*catenin* locus may account for decreased expression in a subset of cases. One of seven prostate cancer cell lines examined had a homozygous deletion of α-*catenin* sequences (Morton *et al.*, 1993) and a lung cancer cell line has been found to have a complete loss of α-catenin expression as a result of localized mutations in both α-*catenin* alleles (Oda *et al.*, 1993). In addition, alterations in β-catenin and plakoglobin expression and phosphorylation have also been noted in some tumor cell lines (Sommers *et al.*, 1994). Furthermore, the protein product of the adenomatous polyposis coli (*APC*) tumor suppressor gene is known to complex with α- and β-catenin, but not with E-cadherin (Rubinfeld *et al.*, 1993; Su *et al.*, 1993; Hulsken *et al.*, 1994). Although the functional significance of the interactions between α- and β-catenin and the APC protein is not yet well understood, the critical role of the APC gene product in tumor suppression in epithelial cells in the gastrointestinal tract is well established (Groden *et al.*, 1991; Nishisho *et al.*, 1991). The interaction between the catenins and an established tumor suppressor gene product lends further support to the proposal that alterations in E-cadherin and catenin function may have a critical role in tumorigenesis.

In the studies described here we have sought to characterize the prevalence of alterations in E-cadherin and α- and β-catenin expression in human breast cancer-derived cell lines and to address the mechanisms underlying their altered expression. We have chosen to examine cell lines in our initial analysis to eliminate ambiguities in studies of protein and RNA expression that might have arisen as a result of the normal cells that are often admixed with neoplastic cells in primary breast cancer specimens. Complete loss or markedly decreased expression of E-cadherin and α-and β-catenin is frequently seen in breast cancer cell lines. The altered expression of E-cadherin and the catenins may result from specific mutations in the genes in some tumors and in others may reflect changes in transcriptional or post-transcriptional regulatory mechanisms. Collectively, the data support the proposal that loss of function in the cadherin-catenin pathway may play a critical role in the pathogenesis of human breast cancer.

Results

Western blot studies of E-cadherin and α- and β-catenin expression

Western blot analyses of E-cadherin, α-catenin and β-catenin expression were carried out on 18 breast carcinoma -derived cell lines (summarized in Table 1). E-cadherin protein was detectable in eight lines, and α-catenin protein was detected at varying levels in 16 lines (Figure 1). No detectable α-catenin protein was seen in two cell lines (lanes 8 and 17). Reduced levels of α-catenin were seen in six lines (lanes 3, 6 and 11–14). β-catenin protein was detected at varying levels in 16 of the cell lines. No detectable β-catenin was seen in two lines (lanes 3 and 14), and decreased levels were noted in six other cell lines (lanes 2, 8, 9, 11–13, 18). In addition, a reactive protein with aberrant migration that may represent a proteolytic breakdown product or a truncated, mutant β-catenin protein product was detected in one line (lane 10).

In an effort to determine if other proteins associated with the membrane cytoskeletal matrix might also display altered expression in the breast carcinoma-derived cell lines, we studied the expression of three

Table 1 E-cadherin, α-catenin, and β-catenin gene and protein expression in breast cell lines

		E-cadherin		*α-catenin*		*β-catenin*		*HER2/Neu*
Cell line#	*Identity*	*RNA*	*Pro*	*RNA*	*Pro*	*RNA*	*Pro*	*Pro*
1	MDA-MB-361	+++	+	++	+++	+++	+++	+++
2	BT-474	++	++	++	+++	+	+	++++
3	ZR-75-30	+/–	–	+++	+	++	–	++++
4	BT-20	++	+	++	+++	+++	+++	+
5	HBL-100	–	–	++	+++	++	++	+
6	DU4475	+	–	+	+	+++	+++	+/–
7	HS 578T	–	–	++	+++	++	+++	+
8	MDA-MB-468	+	+	+	–	+++	+	+
9	ZR-75-1	++	++	++	+++	++	+	++
10	BT-483	++	++	++	+++	++	++	++
11	MDA-MB-435S	–	–	+	+	++	+	+
12	MDA-MB-231	–	–	++	+	++	+	+
13	MDA-MB-453	+/–	–	++	+	++	+	+++
14	SK-BR-3	–	–	+++	+	++	–	+++
15	T-47D	++	++	++	+++	++	+++	+
16	BT-549	–	–	++	+++	++	+++	+
17	MDA-MB-157	–	–	–	–	++	++	+
18	MCF-7	+++	+	++	+++	++	+	+

Relative levels of RNA and protein expression are indicated based on RNase protection studies of gene expression and ECL-Western blot studies of protein expression, with the following scoring system: '–' no detectable expression; '+/–' very low expression; '+' low expression; '++' moderate expression; '+++' high expression; '++++' very high expression

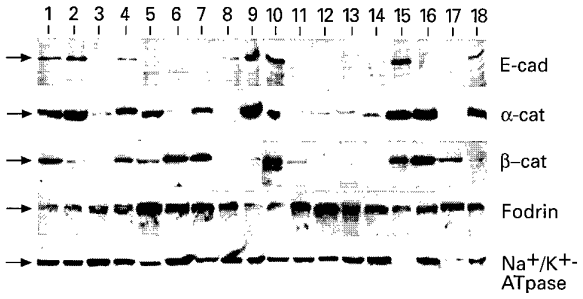

Figure 1 ECL-Western blot studies of E-cadherin and α- and β-catenin expression. Protein lysates were prepared from 18 breast cancer cell lines and 40 μg of protein from each line was loaded for SDS–PAGE on 7.5% gels. Proteins were transferred to Immobilon P membranes, and the membranes were incubated with a specific primary antiserum against E-cadherin, α-catenin, β-catenin, β-fodrin, or Na^+-K^+-ATPase and horseradish peroxidase-labeled secondary antibody reagents. Antibody complexes were detected by ECL. For each protein species studied (e.g., E-cadherin, α-catenin, etc.), although the lysates were electrophoresed on two gels and transferred to two membranes, the ECL exposure times for all 18 lanes are equivalent. The lane numbers correspond to the reference numbers in Table 1

other proteins localized to either the membrane (i.e., α-subunit of Na^+-K^+-ATPase) or the submembrane cortical cytoskeleton (i.e., βII-spectrin or β-fodrin, a spectrin-related protein that complexes with ankyrin and the band 4.1 protein which is thought to stabilize interactions between spectrin and actin). Only relatively subtle differences in β-fodrin levels were noted among the cell lines and most differences appeared to be due to decreased transfer of the gel lanes near the edges of the membrane for this large protein with a relative molecular mass greater than 200 000 (Figure 1 and data not shown). In addition, no apparent differences were noted in the expression of the band 4.1 protein in the cell lines (data not shown). However, two lines were found to have markedly reduced Na^+-K^+-ATPase α-subunit expression (lanes 15 and 17).

RNase protection studies of E-cadherin *and* α- *and* β-catenin *gene expression*

Ribonuclease (RNase) protection studies were carried out in an effort to determine if the levels of E-*cadherin* and α- and β-*catenin* transcripts were correlated with their levels of protein expression in the cell lines. Antisense riboprobes for each gene as well as a control gene (γ-*actin*) were synthesized and incubated with total RNA from each of the 18 cell lines. E-*cadherin* transcripts were detected in 11 lines (Figure 2A). Overall, the relative levels of E-*cadherin* transcripts and protein in the cell lines were well correlated (Table 1, $P < 0.01$). Altered transcriptional regulation and/or mutations interfering with gene expression are likely to account for the concordant decrease in levels of E-*cadherin* transcripts and protein in the affected cell lines.

In contrast to the findings for E-cadherin, the abundance of α- and β-*catenin* transcripts in the cell lines was not well-correlated with their respective protein levels. For example, several cell lines (e.g., lanes 3, 12 and 14) had levels of α-*catenin* transcripts comparable to other lines (Figure 2B), but had very reduced levels of α-catenin protein in the Western blot analysis (Figure 1 and Table 1). The two cell lines lacking α-catenin protein by Western blot analysis had reduced or undetectable levels of α-*catenin* transcripts in the RNase protection analysis (Figure 2B, lanes 8 and 17, respectively). β-catenin protein levels were also not well correlated with transcript levels (e.g., lanes 3, 8, 12, 14 in Figures 1 and 2C; Table 1).

Southern blot and PCR-SSCP analysis of E-cadherin *gene sequences*

To determine if gross rearrangements of E-*cadherin* sequences might account for decreased or undetectable levels of E-*cadherin*, we carried out Southern blot analysis on EcoRI-digested DNA from the cell lines using a full-length E-*cadherin* cDNA probe. Changes in the migration or relative intensity of the detected fragments were seen in two cell lines (SK-BR-3, Figure 3, lane 14; and MDA-MB-468, data not shown). Based on the EcoRI fragments that failed to react with the full-length E-*cadherin* cDNA probe in the analysis of SK-BR-3, the majority of the E-*cadherin* exons in this cell line are affected by homozygous deletion (Figure 3 and data not shown). Consistent with these results, no E-cadherin protein or transcripts were detected in the SK-BR-3 line (Figures 1 and 2A, lane 14). Using an E-*cadherin* cDNA probe corresponding to exon 13–16 sequences in Southern blot analysis, we noted an EcoRI fragment with altered migration in the MDA-MB-468 cell line (data not shown). This cell line

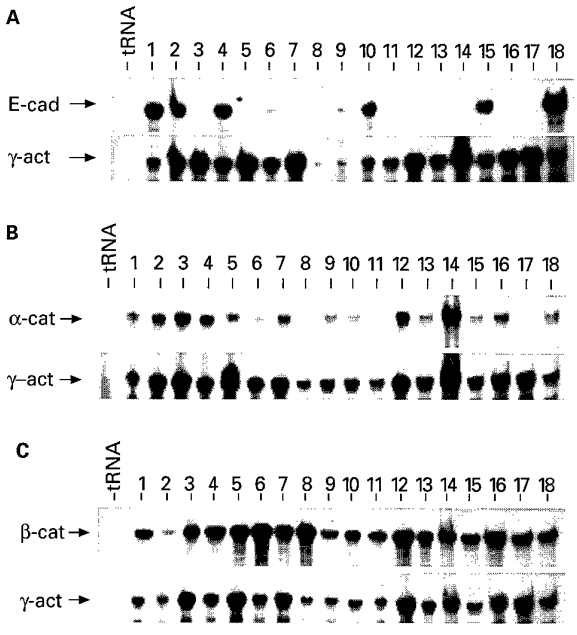

Figure 2 Ribonuclease (RNase) protection assays of E-*cadherin*, α-*catenin* and β-*catenin* gene expression. The lane numbers correspond to the reference numbers for the cell lines in Table 1 and Figure 1, with the exception of a control sample of yeast tRNA. Approximately 5 μg of total RNA from each sample was incubated with 2.5×10^4 c.p.m. of each of the acrylamide/urea gel-purified ^{32}P-labeled anti-sense riboprobes: (**A**) - E-*cadherin* and γ-*actin*; (**B**) - α-*catenin* and γ-*actin*; (**C**) - β-*catenin* and γ-*actin*. The γ-*actin* riboprobe was co-incubated with each of the other riboprobes to control for sample loading and RNA integrity

expressed decreased but roughly concordant levels of E-*cadherin* transcripts and protein. Thus, the altered EcoRI-pattern seen in MDA-MB-468 following Southern analysis with E-*cadherin* cDNA probes is likely to reflect DNA polymorphism rather than mutation. No gross alterations were seen in any of the lines when a full-length α-*catenin* cDNA probe was used for Southern blot analysis of EcoRI-digested DNA from the cell lines (Figure 3 and data not shown).

Because localized mutation in E-*cadherin* sequences have previously been observed in a subset of gastric carcinomas, as well as in some ovarian and endometrial cancers, we carried out a combined polymerase chain reaction and single strand conformational polymorphism (PCR-SSCP) analysis of E-*cadherin* cDNAs obtained from cell lines in which E-*cadherin* transcripts were detected by the RNase protection assay. No sequence alterations were detected in this analysis, suggesting that localized mutations in the E-*cadherin* gene are not common in breast carcinoma cell lines expressing E-*cadherin* transcripts and protein.

Relationship between HER-2/NEU overexpression and E-cadherin expression in breast cancer cell lines

HER-2/NEU overexpression and/or amplification has been noted in a subset of breast cancers, and in several studies overexpression and/or amplification has been shown to be an independent risk factor for disease recurrence (Slamon *et al*., 1987; Press *et al*., 1993; Dougall *et al*., 1994). A recent study has suggested that overexpression of HER-2/NEU in an immortalized human mammary epithelial cell line is associated with an inhibition of E-*cadherin* transcription (D'souza and Taylor-Papadimitriou, 1994). Therefore, we sought to determine if there was a correlation between the levels of HER-2/NEU expression and E-*cadherin* expression in the breast carcinoma-derived cell lines. The relative levels of HER-2/NEU expression were characterized in the cell lines by an ECL-Western blotting approach. We found that there was no apparent correlation between E-*cadherin* expression and HER-2/NEU expression levels (Table 1 and data not shown). It has also been previously reported that E-cadherin expression was reduced but detectable in the SK-BR-3 line, a line with HER-2/NEU amplification and overexpression (D'souza and Taylor-Papadimitriou, 1994). While we confirmed that SK-BR-3 expressed high levels of HER-2/NEU, as we noted above, SK-BR-3 failed to express E-*cadherin* RNA and protein endogenously because of a homozygous deletion involving a large portion of the E-*cadherin* coding sequences.

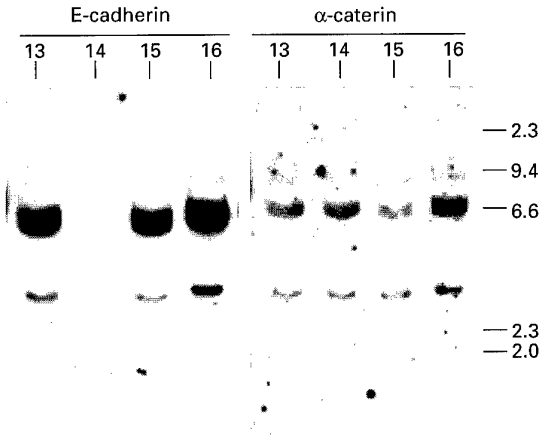

Figure 3 Southern blot analysis of EcoRI-digested breast cancer cell line DNAs with E-*cadherin* and α-*catenin* probes. The lane numbers correspond to the reference numbers for the cell lines in Table 1 and Figure 1. Approximately 10 μg of DNA from each cell line was digested with EcoRI and Southern blot analysis was carried out. The same blot was hybridized to full-length E-*cadherin* and α-*catenin* cDNAs (left and right, respectively). The SK^- BR-3 cell line lost most of the EcoRI fragments detected by the E-*cadherin* cDNA probe. The migration of λ/HindIII size markers in kilobasepairs is indicated at the right

Discussion

Proper inter-cellular interactions are critical to the maintenance of normal cell morphology, differentiation, and growth control. Destabilization or loss of normal cell-cell interactions, as a result of defects in the function of the adherens junction or the submembrane cortical cytoskeleton, may have a critical role in the altered phenotype properties of cancer cells. Data supporting this proposal include the following: (i) the observations of decreased or absent E-cadherin or α-catenin reactivity in immunohistochemical studies of a number of different cancers (Schipper *et al*., 1991; Shimoyama and Hirohashi, 1991a,b; Shiozaki *et al*., 1991; Umbas *et al*., 1992; Brabant *et al*., 1993; Bringuier *et al*., 1993; Dorudi *et al*., 1993; Doki *et al*., 1993; Gamallo *et al*., 1993; Mayer *et al*., 1993; Moll *et al*., 1993; Morton *et al*., 1993; Oka *et al*., 1993; Rasbridge *et al*., 1993; Kadowaki *et al*., 1994; Rimm *et al*., 1995); (ii) the identification of mutations in the E-*cadherin* gene in a subset of gastric and gynecologic cancers (Becker *et al*., 1994; Oda *et al*., 1994; Risinger *et al*., 1994), and α-*catenin* mutations in a prostate cancer and a lung cancer cell line (Morton *et al*., 1993; Oda *et al*., 1993); (iii) the interaction of a known tumor suppressor gene product, APC, with α- and β-catenin (Rubinfeld *et al*., 1993; Su *et al*., 1993; Hulsken *et al*., 1994); (iv) the demonstration that β-catenin is phosphorylated on tyrosine residues either directly or indirectly by known oncogene products, including src, the EGF receptor and met (Hulsken *et al*., 1994); and (v) the demonstration that a transfected E-*cadherin* gene can suppress the invasive properties of some tumor cell lines with decreased or absent endogenous E-cadherin expression (Vleminckx *et al*., 1991).

In the studies described here we have addressed the prevalance of and mechanisms underlying altered E-cadherin and α- and β-catenin expression in breast cancer cell lines. Although previous immunohistochemical studies have demonstrated that E-cadherin and α-catenin immunoreactivity are each decreased or absent in about 50% of primary breast cancers (Shiozaki *et al*., 1991; Gamallo *et al*., 1993; Moll *et al*., 1993; Oka *et al*., 1993; Rasbridge *et al*., 1993; Rimm *et al*., 1995), the mechanisms underlying the altered patterns of immunoreactivity remain quite poorly characterized. In addition, while immunofluorescence studies have been carried out to address β-catenin expression in breast cancer cell lines (Sommers *et al*., 1994), no studies

addressing β-catenin immunoreactivity or expression in primary breast cancers have been presented in the literature. We have chosen to investigate the mechanisms underlying altered E-cadherin and catenin expression in breast cancer cell lines, because of some of the technical difficulties encountered in detailed studies of protein and RNA expression and gene structure and sequence in many primary breast cancers.

As summarized in Table 1, many lines had reduced or undetectable levels of expression of E-cadherin and/or the catenins. In some lines specific mutations are likely to account for the altered expression patterns. For example, a homozygous deletion of a large portion of the E-*cadherin* gene was noted in one cell line. Nevertheless, with the exception of this line, the mechanisms underlying the diminished levels of E-*cadherin* gene and protein expression noted in nearly half of the lines remain relatively poorly understood. Although the possible mechanisms include localized mutations in the E-*cadherin* gene that interfere with synthesis, processing, or stability of its transcripts, E-*cadherin* expression may be decreased as a result of specific defects in upstream regulatory pathways or transcription factors that control its expression. Indeed, consistent with this notion, previous studies suggest that E-*cadherin* promoter activity may be correlated with endogenous E-*cadherin* expression in some breast cancer cell lines (Behrens *et al*., 1991). Specific factors regulating E-*cadherin* promoter activity have not yet been well defined. Although a recent study suggested HER-2/NEU overexpression in an immortalized, non-tumorigenic breast cell line was associated with inhibition of E-*cadherin* transcription (D'souza and Taylor-Papadimitriou, 1994), in our studies, we were unable to demonstrate a correlation between HER-2/NEU overexpression and E-cadherin expression in the breast cancer cell lines.

While the relative abundance of E-*cadherin* transcripts and protein correlated well in the cell lines (Table 1), the relative levels of catenin gene and protein expression were often discordant. Specifically, although two lines with no detectable α-catenin protein expressed very reduced or undetectable levels of α-*catenin* transcripts, the majority of lines with reduced α-catenin protein had no consistent differences in α-*catenin* transcript levels. Similarly, most lines with reduced but detectable levels of β-catenin protein had no clear-cut differences in the abundance of β-*catenin* transcripts when compared to lines with abundant levels of β-catenin protein. Given that previous studies have identified α-*catenin* mutations in some cancer cell lines (Morton *et al*., 1993; Oda *et al*., 1993), a subset of the breast cancer lines with altered expression of α- or β-catenin protein may have specific mutations in the corresponding gene. An alternative, but not mutually exclusive, explanation is that an assortment of alterations in post-transcriptional and post-translational regulation of catenin expression may account for the decreased levels of catenin proteins in the cancer cell lines.

While immunohistochemical studies suggest that ductal and lobular breast cancers have some differences in the prevalence of altered E-cadherin and α-catenin expression (Ochiai *et al*., 1994), it was not clear a priori whether cell lines derived from breast cancers of differing histopathological types would display distinctly different patterns of expression. Eight of the cell lines in this study were reportedly derived from tumors with ductal histopathology (ATCC:BT-474, ZR-75-30, Hs578t, ZR-75-1, BT-483, MDA-MB-435s, T-47D, BT-549). Alterations in E-cadherin or catenin expression were seen in only a subset of these eight lines and were also seen in a similar percentage of the other lines for which the histology of the primary tumor was not noted. Thus, alterations in E-cadherin and catenin expression do not appear to be restricted to breast cancers of a particular histologic subtype.

The adhesive capacity of breast cancer-derived cell lines in Matrigel has been correlated with their expression of E-cadherin and vimentin (Sommers *et al*., 1991; Thompson *et al*., 1992). Data from those studies suggested E-cadherin expression correlated with the ability of a cell line to form spherical colonies or non-invasive clusters in Matrigel and that cell lines with high levels of vimentin expression formed invasive colonies. Nevertheless, these *in vitro* growth properties may not fully reflect appropriate function of the cadherin-catenin pathway, as one cell line (MDA-MB-468) that failed to express α-catenin protein and another cell line (SK-BR-3) lacking E-cadherin and β-catenin protein formed spherical colonies/non-invasive clusters in matrigel (Thompson *et al*., 1992).

In summary, the data presented here suggest that alterations in E-cadherin and α- and β-catenin expression are common in breast cancer cell lines. Although we cannot exclude the possibility that alterations in E-cadherin and catenin expression and gene structure may have arisen during the establishment and subsequent passage of the cell lines, the prevalence of altered expression of E-cadherin and α-catenin in the breast cancer cell lines appears to be relatively well-correlated with the results obtained from previous immunohistochemical studies of primary breast cancers. Additional studies will be necessary to further elucidate what appears likely to be a complex assortment of mutational and altered regulatory mechanisms underlying the alterations in E-cadherin and catenin expression in breast cancer cells. Moreover, while these data provide further support for the proposal that defects in the E-cadherin-catenin pathway may be a critical and necessary step in the generation of advanced breast cancer cells, definitive functional studies will ultimately be required to establish the relationship and significance of our observations to the altered phenotypic properties of breast cancer cells observed *in vivo*.

Materials and methods

Cell lines

All cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in the recommended growth media. Cultures were incubated at 37°C with 5% CO_2, except for cultures maintained in Lebovitz's L-15 media which were grown at 37°C without CO_2. DNA, RNA, and protein were isolated from cultures at 75–95% confluence.

Western blot analysis

Cells were washed and then lysed in RIPA buffer [25 mM Tris-buffered saline (pH 8) with detergents (1% deoxycho-

late, 0.1% sodium dodecyl sulfate, 1% nonidet P-40) supplemented with 10 μg ml^{-1} phenylmethylsulfonyl fluoride (PMSF), 50 μg ml^{-1} antipain, 5 μg ml^{-1} aprotinin, and 2 μg ml^{-1} leupeptin (all protease inhibitors purchased from Sigma Chemical Co., St. Louis, MO). Protein concentrations were determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL), using bovine serum albumin to generate a standard curve. Forty micrograms of total protein per sample was separated by electrophoresis on SDS/polyacrylamide gels and transferred to Immobilon P membranes (Millipore, Bedford, MA) by semi-dry electroblotting (Tansblot, Bio-Rad, Hercules, CA). Western blot analysis was carried out using affinity-purified polyclonal rabbit and rat or mouse monoclonal antisera as primary antibodies and horseradish peroxidase-conjugated goat anti-rabbit, goat-anti-rat or goat-anti-mouse immunoglobulin antibodies (Pierce) as secondary reagents. E-cadherin was detected with a rat monoclonal antibody DECMA-1 (Sigma). A polyclonal rabbit antiserum YR4 against α-catenin was generated by immunization with a bacterial recombinant protein containing the carboxy-terminal 447 amino acids of α-catenin fused to glutathione S-transferase (GST) (Rimm *et al*., 1995). A rabbit polyclonal antisera against β-catenin was generated by immunization with a bacterial recombinant GST fusion protein containing full-length β-catenin sequence (D Rimm and E Koslov, unpublished observations). A polyclonal rabbit antiserum against βII-spectrin (β-fodrin) was generated by immunization with a bacterial recombinant GST fusion protein containing the carboxy-terminal third of human β-fodrin (SP Kennedy and JS Morrow, unpublished observations). The α-subunit of Na^+-K^+-adrenosine-triphosphatase (Na^+-K^+-ATPase) was detected using mouse monoclonal antibody C464.6 (a gift from Dr M Kashgarian, Dept. of Pathology, Yale University) which has been previously described (Kashgarian *et al*., 1985). Band 4.1 protein was detected with a polyclonal rabbit antiserum raised against native band 4.1 protein purified from a red cell ghost lysate (Croall *et al*., 1986). HER-2/Neu protein was detected by rabbit polyclonal antiserum Ab-1 (Oncogene Science, Uniondale, NY). Detection of antibody complexes was carried out with the Enhanced Chemiluminescence (ECL) Western Blot Kit (Amersham, Arlington Heights, IL) and subsequent exposure to Hyperfilm (Amersham). Western blot data shown in Figure 1 are representative of results obtained from studies performed two or more times.

Ribonuclease protection assay

Total RNA was isolated as described previously (Chomczynski and Sacchi, 1987) or using Trizol reagent (Gibco/BRL Life Technologies). Radiolabeled antisense riboprobe transcripts were prepared from plasmid constructs using T7 or T3 RNA polymerases (GibcoBRL/Life Technologies) or SP6 (New England Biolabs, Bedford, MA) and ^{32}P-CTP. Antisense riboprobes were generated from the following cDNA fragments: (i) E-*cadherin* – a 565 bp fragment containing 438 nucleotides of carboxyl-terminal coding sequences and 127 nucleotides of 3′ untranslated sequences; (ii) α-*catenin* – a 498 bp fragment corresponding to codons 449–615; (iii) β-*catenin* – a 635 bp fragment corresponding to codons 363–575; and (iv) γ-*actin* – a 275 bp fragment derived from the 3′ coding region of the cDNA. Transcripts were purified by electrophoresis, and 2.5×10^4 c.p.m. of each transcript was incubated overnight at 48°C with 5 μg of total RNA in hybridization solution [80% deionized formamide; 40 mM 1,4-piperazinediethane sulfonic acid (PIPES), pH 6.4; 400 mM NaCl; 1 mM EDTA]. The γ-*actin* transcript was co-incubated with the E-*cadherin*, α-*catenin* or β-*catenin* transcripts to control for RNA integrity and loading. Ribonuclease digestion was then carried out for 1 h at 30°C using 7.5 units of RNase T2 (Gibco BRL/Life Technologies, Grand Island, NY) in 250 μl of digestion buffer (50 mM sodium acetate, pH 4.4; 100 mM NaCl; 10 mM EDTA). RNA was precipitated with isopropanol, resuspended in RNA loading buffer (90% deionized formamide; 10 mM EDTA; 0.2% bromophenol blue; 0.2% xylene cyanol), heated for 3 min at 90°C and electrophoresed on a sequencing gel. After drying the gel, autoradiography was carried out with intensifying screens and Hyperfilm (Amersham) at –80°C. Data shown in Figure 2A–C are representative of results obtained in RNase protection assays performed two or more times.

Southern analysis

High molecular weight genomic DNA was isolated by incubation of cell pellets in 1.0% sodium dodecyl sulfate (SDS), 0.5 mg ml^{-1} proteinase K (Gibco BRL/Life Technologies) at 48°C for 24–48 h. After two extractions with phenol:chloroform:iso-amyl alcohol (50:49:1) and one extraction with chloroform:iso-amyl alcohol (49:1), DNA was recovered by ethanol precipitation. DNA concentrations were determined using a diphenylamine assay (Shatkin, 1969). For Southern analysis, 10 μg of genomic DNA was digested with EcoRI (Gibco BRL/Life Technologies), precipitated, and electrophoresed on 1.0% agarose gels. Transfer to Zeta-probe membranes (Bio-Rad) was performed using a positive pressure blotting apparatus (Posiblotter, Stratagene, San Diego, CA). Hybridization of the filters to E-*cadherin* and α-*catenin* cDNA fragments was carried out as described (Reale *et al*., 1994). After post-hybridization washing, filters were exposed to Hyperfilm (Amersham) with intensifying screens at –80°C.

RT-PCR SSCP analysis

cDNA was prepared from DNase I-treated total RNA using random hexamers and Superscript reverse transcriptase (Gibco BRL/Life Technologies). E-*cadherin* sequences were then amplified by 35 cycles of polymerase chain reaction (PCR), with each cycle consisting of 30 s at 94°C, 45 s at 56°C, and 1 min at 72°C (last cycle 5 min). The PCR was carried out in the presence of 1.0 μCi [α-^{32}P]dCTP using standard reaction reagents and the following E-cadherin oligonucleotide pairs: pair 1 - ECAD131: 5′ - GAGAGAGGCCGCGTCCTGGGCA - 3′ and ECAD513: 5′ - CCAGGTTTTTAGGAAATGGGCC-3′; pair 2 - ECAD431: 5′-CCTCAGAAGACAGAAGAGA-GAC - 3′ and ECAD958: 5′ - CCTGTGTTCCTGTTAATG-GTG-3′; pair 3: ECAD822: 5′-ACCTCTGTGATGGAGG TCACAG and ECAD1118: 5′-GGGATTGAAGATCGGA GGATTATC-3′; pair 4: ECAD1003: 5′-CTACGTATACC-CTGGTGGTTCA-3′ and ECAD1365: 5′-CCACATTCGT-CACTGCTACG - 3′; pair 5: ECAD1473: 5′ - TCCGAGGA CTTTGGCGTGGGC-3′ and ECAD1790: 5′-GAATA-TAGTTCGAGGTTCTGGTAT-3′; pair 6: ECAD1731: 5′-CTGCTGATCCTGTCTGATGTG-3′ and ECAD2113: 5′-GCAGGAATTTGCAATCCTGCTTCG-3′; pair 7: ECAD 2080: 5′ - CACAGCCTGTCGAAGCAGGATTGC - 3′ and ECAD2524: 5′-CTCAGACTAGCAGCTTCGGAACCGC-T-3′; and pair 8: ECAD2490: 5′-TATGAAGGAAGCGG-TTCCGAA-3′ and ECAD2688: 5′-ACGCTGATTTCTG CATTTCTGCAC-3′. Following amplification, 1.5 μl from each 10 μl reaction was diluted into 3.5 μl formamide sequencing stop solution, heated to 90°C for 5 min, quickly chilled on ice, and then loaded immediately onto 5% Long Ranger (AT Biochem, Malvern, PA)/10% glycerol/0.6% TBE buffer sequencing type gels and electrophoresed at 15 W for 7 h at room temperature. The gel was dried and autoradiography was carried out at –80°C with intensifying screens and Hyperfilm (Amersham).

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Inactivation of *DCC* But Not the *DCC*-Related Gene Neogenin in Cancer[1]

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Abbreviations: DCC - deleted in colorectal cancer; NGN - human neogenin; FN III - fibronectin type III, bp - base pair; kb - kilobase pair; RNase -ribonuclease; RT-PCR - reverse transcription polymerase chain reaction; VSV-G - vesicular stomatitis virus glycoprotein; kDa - kilodalton; ECL - enhanced chemiluminescence

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ABSTRACT

DCC (deleted in colorectal cancer), a candidate tumor suppressor gene located in chromosome band 18q21.2, encodes a transmembrane protein of 1447 amino acids. Neogenin, a protein with nearly 50% amino acid identity to DCC, was recently identified because of its dynamic expression in the developing nervous system and gastrointestinal tract of the chicken. To explore a role for the human neogenin (*NGN*) gene in cancer, we have isolated cDNAs for two alternatively spliced forms of *NGN*, encoding proteins of 1461 and 1408 amino acids. Fluorescence in situ hybridization studies (FISH) localized *NGN* in chromosome band 15q22, a region infrequently affected by alterations in cancer. *NGN* transcripts were detected in all adult tissues studied. In contrast to the frequent loss of *DCC* expression, no alterations in *NGN* expression were observed in the cancers studied, including glioblastoma, medulloblastoma, neuroblastoma, colorectal, breast, cervical, and pancreatic cancer cell lines and xenografts. Based on their sequence conservation and similar expression during development, *DCC* and *NGN* may have related functions. However, while its localization at 18q21 and frequent loss of expression in cancers are consistent with the proposal that *DCC* is a candidate suppressor gene, the chromosomal location and ubiquitous expression of *NGN* in various types of human cancer suggest it is unlikely to be a tumor suppressor gene.

INTRODUCTION

Enormous progress has been made in describing genetic alterations in human cancer cells (1-3). The identification of more than 50 different oncogenes has been facilitated by the prior isolation of viral oncogenes, the detection and characterization of translocation breakpoints in cancer cells, and the ability of some oncogenes to promote tumorigenic growth when transferred to nontumorigenic recipient cells. Despite much recent attention, only about fifteen tumor suppressor and candidate tumor suppressor genes have been molecularly cloned (2,3). Results from several independent experimental approaches, however, suggest that a sizeable number of suppressor genes await discovery (2-15). One potential shortcut to the identification of novel tumor suppressor genes might be realized through study of genes sharing close sequence similarity with known or candidate tumor suppressor genes.

The existence of a tumor suppressor gene(s) on chromosome 18q was first suggested by frequent allelic losses of 18q in colorectal cancers (5,16). Subsequent studies identified the *DCC* (deleted in colorectal cancer) gene at 18q21.2 as a candidate suppressor gene (17). *DCC* is an enormous gene spanning greater than 1.35 million base pairs (bp), and it is expressed in most adult tissues, albeit at very low levels (17-20). *DCC* expression is reduced or absent in the majority of colorectal cancers, though specific somatic mutations in *DCC* have only been identified in a subset of cases (17,18,21). Loss of *DCC* expression has also been seen in some cancers of the breast, pancreas, endometrium, prostate, and brain, as well as male germ cell cancers, leukemias, and neuroblastomas (21,22). Mutations in *DPC4*, a candidate pancreatic tumor suppressor gene from 18q21.1, have been found in 40-50% of pancreatic cancers, but recent studies suggest that it is infrequently inactivated in other tumors (23-26). Thus, *DCC* remains a strong candidate for the suppressor gene inactivated by 18q allelic loss in many colorectal and other cancers.

DCC encodes a 1447 amino acid transmembrane protein with four immunoglobulin like and six fibronectin type III like extracellular domains, a single membrane spanning region, and a

325 amino acid cytoplasmic domain (19,20). Using sensitive assays, *DCC* transcripts have been detected in most adult tissues, though highest expression is seen in the developing brain and neural tube (17,19,20,27-29). A protein with roughly 50% amino acid identity to DCC, termed neogenin, was identified because of its dynamic pattern of expression in the developing nervous system and gastrointestinal tract of the chicken (30). Specifically, neogenin was induced in neural cells immediately prior to cell cycle withdrawal and terminal differentiation. To test the hypothesis that neogenin might be a candidate suppressor gene, we have cloned the human neogenin (*NGN*) gene. Here, we present further evidence that *DCC* expression is frequently lost in cancer. In contrast to *DCC*, based on its pattern of expression in cancer cells and its chromosomal location, *NGN* appears unlikely to be a tumor suppressor gene.

MATERIALS AND METHODS

Cloning of *NGN*. A 312 basepair (bp) polymerase chain reaction (PCR) product corresponding to a human expression sequence tag (T07322) with 85% identity to chicken neogenin was amplified from an oligo-dT primed human fetal brain library (Stratagene Cloning Systems, La Jolla, CA) using two oligos (5'-TTACGCCATTGGTTATG-3' and 5'-CACCATCAGGATTAC-GTG-3') derived from the ends of the sequence tag. The PCR product was labeled with ^{32}P-dCTP by random priming and used to screen the fetal brain library. Approximately 2 X 10^6 plaques were lifted onto Hybond N+ nylon filters (Amersham, Arlington Heights, IL). The filters were hybridized and washed as described (31). A total of 19 independent clones were isolated by multiple rounds of hybridization selection. Phagemids were rescued by in vivo excision with the ExAssist/SOLR system provided with the library.

DNA sequencing. Both strands of overlapping, double-stranded phagemid clones, containing the entire open reading frame of *NGN* (GenBank #U61262), were sequenced in their entirety using a combination of external and internal primers and exonuclease III/mung bean nuclease deletions (Stratagene). Plasmid DNA was prepared using Qiagen Spin Plasmid Kit (Qiagen, Inc.,

Chatsworth, CA) and sequenced by the dideoxy chain termination method using Sequenase 2.0 (U.S. Biochemical Corp., Cleveland, OH) and a modified protocol (32). Sequencing reactions were electrophoresed on 6% polyacrylamide (19:1 acrylamide:bis-acrylamide)/8.0 M urea/1 X TBE gels. After drying, the gels were exposed to X-OMAT film (Eastman Kodak, Rochester, NY).

***NGN* expression constructs.** Full-length cDNAs encoding each of the two alternatively spliced forms of *NGN* were constructed from the overlapping fetal brain cDNAs. PCR was used to fuse a vesicular stomatitis virus glycoprotein (VSV-G) epitope tag (YTDIEMNRLGK) to the carboxy -terminus of each of the two full-length *NGN* cDNAs. The modified cDNAs were sequenced to verify that no errors had been introduced. The tagged cDNAs were subcloned into the pCDNA3 mammalian expression vector (Invitrogen Corp., San Diego, CA).

Western blot analysis. Transfections of Cos-1 cells (American Type Culture Collection [ATCC], Rockville, MD) were performed with Lipofectamine (Gibco BRL Life Technologies, Gaithersburg, MD) per the manufacturer's instructions. Protein extracts were prepared from the cells 48 hrs after transfection as previously described (28,33). Following electrophoresis on an 8% SDS-polyacrylamide gel and transfer to Immobilon-P membranes (Millipore, Bedford, MA) with a semidry electroblotter (Bio-Rad, Hercules, CA), tagged proteins were detected with a polyclonal rabbit anti-VSV-G antiserum (MBL International, Watertown, MA) and a donkey anti-rabbit IgG coupled to horseradish peroxidase (Pierce Biochemicals, Rockford, IL). Antibody complexes were detected by enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, IL) and subsequent exposure to Kodak X-OMAT film.

P1 clone isolation and fluorescence in situ hybridization (FISH). The P1 library (DMPC-HFF#10) was screened by Genome Systems, Inc. (St. Louis, MO) with two *NGN* primers derived from sequences at the end of the coding region and the downstream 3' untranslated region (nucleotides 4465-4645; sense oligo 5'-GAGATGGCCCACCTGGAAGGAC-3' and

antisense oligo 5'-GTCTGCTGGCTGATTCTGTGTT-3'). Three P1 clones were isolated (DMFC-HFF#1-113-H12, -531-A11, and -1421-D6). Purified DNA from clone #113-H12 was labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN) by nick translation. Phytohemagglutinin-stimulated human peripheral blood lymphocytes from a normal donor were used as the source of metaphase chromosomes. Labeled DNA was hybridized overnight at 37°C to fixed metaphase chromosomes in a solution containing sheared human DNA, 50% formamide, 10% dextran sulfate, and 2X SSC. Specific hybridization signals were detected by incubating the slides with fluorescein-conjugated sheep anti-digoxigenin antibodies (Boehringer Mannheim). The chromosomes were then counterstained with 4,6-diamidino-2-phenylindole (DAPI) and analyzed. Assignment of *NGN* to chromosome 15 was confirmed by co-hybridization of the *NGN* P1 clones and a biotinylated chromosome 15 centromere-specific probe (D15Z) (Oncor, Inc., Gaithersburg, MD). In this case, probe signals were detected by incubating the slides with fluorescein-conjugated sheep anti-digoxigenin antibodies as well as a Texas red avidin conjugate (Vector Laboratories, Burlington, CA). The *NGN* gene was further localized on chromosome 15 by comparing the position of fluorescein signals to chromosome landmarks, such as the centromere, telomere, and heterochrome and euchromatin boundaries (34).

RNA isolation. Brain tumor xenografts were established from primary human glioblastomas and medulloblastomas and propagated in nude mice as previously described (35). Pancreatic xenografts were established from pancreatic adenocarcinomas (9). Human neuroblastoma cell lines SJNB-7, -8, -10, -11, and -17 were derived from advanced stage primary tumors at St. Jude Children's Research Hospital. All other cell lines were purchased from ATCC. Total RNA was isolated from minced brain tumor xenograft tissues or pelleted tumor cells, using Trizol reagent (Gibco BRL Life Technologies) or the RNAgents RNA isolation system (Promega, Madison, WI).

Northern analysis. Northern blots of normal human adult tissues (approximately 2 μg of poly(A+) RNA loaded per lane) were purchased from ClonTech (ClonTech Laboratories, Inc.,

Palo Alto, CA). Hybridizations were performed according to the manufacturer's instructions using a 486 bp ^{32}P-labeled *NGN* cDNA probe (corresponding to amino acids 330-491) or a 4.35 kb ^{32}P-labeled *DCC* cDNA probe (19). Following hybridization, blots were washed with 2X SSC/0.5% SDS for 45 min at room temperature, with a subsequent increased stringency wash of 0.1X SSC/0.1% SDS for 30 min at 50°C. Blots were stripped per the manufacturer's instructions, and reprobed with a ^{32}P-labeled 2.0 kb cDNA fragment of β-actin, provided by ClonTech.

Ribonuclease protection assay. Ribonuclease (RNase) protection assays were performed essentially as described (28,33). A *NGN* riboprobe was generated from pAMP1-T07322, a plasmid containing a 312 bp *NGN* cDNA fragment (corresponding to amino acids 771-873). The *DCC* riboprobe has been previously described (33). To control for loading, γ-actin and β-actin riboprobes were used. The γ-actin riboprobe has been described (33), and the β-actin riboprobe was prepared from the pTRI-β-actin-125-human plasmid construct (Ambion, Inc., Austin, TX). Probes were labeleled with ^{32}P-CTP, and following purification through an acrylamide gel, 1.0 X 10^6 cpm of the *NGN* and 2.0 X 10^5 cpm of the γ-actin or β-actin riboprobe were incubated with 10 μg of RNA. Similarly, 1.0 X 10^6 cpm of the *DCC* riboprobe and 2.0 X 10^5 cpm of the γ-actin riboprobe were hybridized overnight with 20 μg of RNA. Non-hybridizing sequences were digested with RNase T2 (Gibco BRL Life Technologies). Protected fragments were recovered by ethanol precipitation and electrophoresed on a denaturing polyacrylamide sequencing gel. After drying the gel, autoradiography was carried out with X-OMAT film and intensifying screens.

RT-PCR assay. Total RNA was treated with 2 units of RNase-free DNase (Boehringer Mannheim). First-strand cDNA was prepared from 3 micrograms of RNA using AMV reverse transcriptase (Promega) and random hexamers. One tenth of the cDNA was used for each PCR with primer pairs derived from the human *DCC* and *NGN* sequences. Extracellular domain *NGN* primers (corresponding to amino acids 329-491) were NGN329S -5'-TTGAAGCTCAAGCAGA-GCTTACAG-3' and NGN491A - 5'-GACTGGTATTCTCAACACGTTCC-3'. *NGN*

cytoplasmic domain primers (amino acids 1128-1239) were NGN1128S - 5'-GTACCCGTCGT-ACCACCTCTCACC-3' and NGN1239A - 5' CATCATTTTTGGTCTCATTCCTCG-3'. *DCC* extracellular domain primers (amino acids 93-221) were DCC902S - 5'-CAAATGGGTCTCTG-CTGATAC-3' and DCCEX3A - 5'-TCTTGAGCTGGCTGGATTTCGAGC-3'. *DCC* cytoplasmic domain primers (amino acids 1110-1309) were DCK3090S - 5'-CACAGTGCTG-GTAGTGGTCAT-3' and DCK4504A - 5'-TTGGGTTGATGGTCCTTCACTCAC-3'. Amplifications were performed using the following conditions: hotstart followed by 35 cycles of 94°C x 45 sec, 55°C x 45 sec, and 72°C x 2.5 min. One-fifth of each reaction was electrophoresed on 1.2% agarose gels and visualized with UV light following ethidium bromide staining. The identities of the *DCC* and *NGN* products were confirmed by Southern transfer and hybridization with their respective ^{32}P-labelled cDNA probes. A set of β-actin primers was used to independently confirm the first strand cDNA reaction. For all samples studied, the results with the two sets of *NGN* primers were concordant. Similarly, concordant results were obtained with the two sets of *DCC* primers.

RESULTS AND DISCUSSION

Given the frequent loss of *DCC* expression in cancer, we sought to determine if *DCC*-related genes might also be inactivated in cancer. A human expression sequence tag (T07322) with over 85% nucleotide identity to chicken neogenin, a *DCC*-related gene, was identified in the GenBank database. Using this sequence as a hybridization probe, we isolated cDNA clones from a fetal brain library spanning 5297 bp and containing the entire human neogenin (*NGN*) open reading frame (Fig. 1). Two alternatively spliced forms of neogenin were previously identified in the chicken; the isoforms differ by the presence or absence of a 159 bp sequence in the neogenin cytoplasmic domain. We identified both alternatively spliced forms. NGN protein products of 1461 and 1408 amino acids were predicted from the sequences (Fig. 1).

The extracellular domain of human NGN displayed features common to members of the neural cell adhesion molecule (N-CAM) family, with four immunoglobulin like and six fibronectin type III (FN III) like domains. Eight potential asparagine (N)-linked glycosylation sites (N-X-S/T) were identified in the approximately 1100 amino acid extracellular region (Fig. 1). A single hydrophobic membrane-spanning sequence was found. In the long isoform of *NGN*, a cytoplasmic domain of 338 amino acids with 14 potential phosphorylation sites was observed (Fig. 1). Three of the sites are lost in the alternatively spliced short *NGN* isoform. Overall, the predicted amino acid sequence of human NGN was 86% identical to the chicken neogenin sequence, with the greatest similarity seen in the FN III and cytoplasmic domains. Comparison of human NGN and DCC revealed that the proteins had identical domain structure and roughly 50% identity at the amino acid level. The cytoplasmic sequences of NGN and DCC were less well-conserved, with only about 37% identity at the amino acid level. However, their cytoplasmic domains do not share extensive similarity with other proteins in the database.

To demonstrate that the *NGN* cDNAs encoded proteins, Western blot analysis was performed with lysates of Cos-1 cells that had been transiently transfected with expression constructs encoding the two alternatively spliced forms of *NGN*. To facilitate their detection, vesicular stomatitis virus glycoprotein (VSV-G) epitope tags were fused to the carboxy-termini of each protein. Both cDNAs encoded proteins migrating at about 190 kDa, with the shorter NGN isoform migrating slightly more rapidly (Fig. 2). Given the eight potential N-linked glycosylation sites in the NGN extracellular domain and the fact that DCC migrates at 175-190 kDa (19,20), the apparent molecular masses were in good agreement with those predicted from the sequences.

Data from allelic loss studies of various cancers suggest that a number of chromosomal regions contain novel tumor suppressor genes awaiting identification (3-15). We utilized fluorescence in situ hybridization to localize *NGN* to chromosome 15q22 (Fig. 3). In previous studies, allelic losses of 15q have been infrequently observed in cancer (4-15). One recent study

suggested that 15q allelic losses were common in metastatic cancers of the breast, colon, and lung. However, the losses were restricted to proximal 15q and did not include the 15q22 region (36). Hence, based on its location and the allelotype studies carried out thus far, *NGN* does not appear to be a frequent target for allelic losses in cancer.

Using Northern blot analysis, *NGN* transcripts of about 7.5 and 5.5 kb were detected in all normal adult tissues studied (Fig. 4A and data not shown). In previous studies, *DCC* transcripts have been detected in most normal adult tissues (17,20). However, their very reduced abundance has often necessitated very sensitive detection methods, such as reverse transcription polymerase chain reaction (RT-PCR). Although previous studies have suggested that *DCC* transcripts were only detectable by Northern blot analysis in adult brain (17), we were able detect *DCC* transcripts of about 10 kb and/or 7 kb in most all adult tissues studied (Fig. 4B and data not shown). Of note, in testis, we also detected relatively high levels of *DCC* transcripts of altered size (5.5 and 4.0 kb), which have not been characterized in detail.

We carried out ribonuclease (RNase) protection studies to assess the abundance of *NGN* and *DCC* transcripts in cancers. In glioblastomas, *NGN* expression was detected in all specimens studied (Fig. 5, Table 1), though *DCC* expression was absent or very reduced in upwards of 40% (Table 1), confirming previous results (33). *NGN* expression was also detected in all 7 medulloblastoma xenografts studied, but *DCC* expression was absent in 2 of the 7 tumors (Table 1). In additional RNase protection and RT-PCR studies, *NGN* expression was detected in all other cancer lines studied, including neuroblastoma, breast, colorectal, pancreatic, and cervical cancers (Figs. 5 and 6, Table 1). In contrast, *DCC* expression was not detectable in the vast majority of these lines (Fig. 6 and Table 1).

In agreement with the results of Hohne et al. (37) and in contrast to those of Barton et al. (38), we failed to detect *DCC* transcripts in most pancreatic cancers studied (Fig. 6 and Table 1).

However, while our results were in general agreement with the Hohne et al. study, we noted discrepancies in the *DCC* expression results for two of the cell lines studied by both Hohne et al. and our group (i.e., CAPAN2 and Panc1). It is possible that these discrepancies may have resulted from confusion in the identities of the cell lines. Based on our results, it was interesting to note that *DCC* expression was detected in a cell line with a *DPC4* mutation, but no *DCC* expression was detected in 5 of the 6 pancreatic cancer lines lacking *DPC4* mutations (23,24). Because upwards of 50% of pancreatic cancers with 18q allelic loss lack detectable *DPC4* mutations, we propose that *DCC* may be inactivated by 18q allelic loss and other mechanisms in a sizeable fraction of pancreatic cancers.

Loss of *DCC* expression in cancers does not establish that *DCC* is a tumor suppressor gene. Nonetheless, the data are consistent with that possibility. Unfortunately, because of its extremely large size, definitive examination of the *DCC* locus for alterations affecting its expression in cancer cells, such as somatic mutations or increased methylation of its regulatory sequences, was not possible. The chromosomal location and ubiquitous expression of *NGN* in cancer suggest that it is unlikely to be a suppressor gene. The findings presented here also imply that, despite their extensive sequence similarity, the specific functions of *DCC* and *NGN* in cell growth regulation and cancer are likely to be distinct.

Recent studies of genes regulating cell migration and axon guidance in the developing nervous system have provided interesting new clues into *DCC* and *NGN* function. The C. elegans *unc-40* gene encodes a transmembrane protein with identical domain structure to DCC and NGN and about 25% amino acid identity with each (39). Though it may also have other functions, *unc-40* is necessary for the appropriate circumferential migration of a subset of cells and axons in the developing nematode (40). *unc-40* is believed to function in the same pathway as another gene termed *unc-6*(40,41). *unc-6* encodes a secreted protein bearing significant similarity to the amino-terminal region of the B2 chain of laminin, an extracellular matrix protein (42), and two vertebrate

homologues of *unc-6*, termed netrin-1 and netrin-2 have recently been identified (43,44). The netrin proteins were initially identified and purified because of their ability to promote the outgrowth of commissural axons, but they also appear to function as chemoattractants for commissural axons (44,45). Given the predicted similarity of UNC-40 to DCC and NGN, the findings raise the interesting possibility that *DCC* and *NGN* may play important roles in mediating directional cell migration in the developing nervous system.

What role, if any, would *DCC* , *NGN*, and the netrins be expected to play in other tissues? Moreover, how would loss of *DCC* function contribute to the altered phenotype of cancer cells, particularly if *NGN* function is retained in the cells? As shown above, *DCC* and *NGN* are both expressed at low levels in virtually all adult tissues. We have cloned the human netrin-1 gene and have found it is expressed in all adult tissues surveyed (Meyerhardt J, et al., unpubl. observ.). In addition, netrin-1 transcripts can be detected in many human cancer cell lines, including those derived from colorectal tumors (Meyerhardt J, et al., unpubl. observ.). The effects of netrins on epithelial cells are poorly understood. However, it is tempting to propose that netrins may provide growth inhibitory or differentiation cues to epithelial cells. Given the substantial differences between the DCC and NGN cytoplasmic sequences, cancer cells that have lost *DCC* function may fail to respond appropriately to netrin signals, despite retaining *NGN* expression. Alternatively, *DCC* alterations may contribute to defects in the migratory properties of cancer cells or their failure to respect tissue boundaries. Indeed, such an effect might account for the apparently more aggressive and metastatic growth properties of some cancer cells lacking *DCC* expression (21,22).

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Table 1. ***NGN*** **and** ***DCC*** **expression in tumor xenografts and cell lines**[1]

Xenograft/Cell Line	*NGN*	*DCC*
Glioblastomas		
54	++	+/-
245	++	++
259	+++	-
270	++	+/-
317	++	+
320	+++	-
368	++	++
397	++	-
398	+++	++
408	+++	++
409	++	+
443	+++	+
493	++	-
542	+++	+
561	++	+
566	++	-
640	++	+/-
Medulloblastomas		
341	++	+
384	+++	-
425	+++	+
487	++	-
511	+++	+
556	+++	+
690	+++	++
Neuroblastomas		
SJNB-7	++	+/-
SJNB-8	+++	+/-
SJNB-10	++	-
SJNB-11	++	-
SJNB-17	++	+
IMR32	++	++
Colorectal		
DLD1	+++	+/-
LoVo	+++	+
WIDR	+++	-
Hct116	+++	-
RKO	+++	+
Breast		
SKBR3	+++	-
MDA-MB-361	+++	-
MDA-MB-231	+++	-
Pancreatic[2]		
AsPC1	+++	+
CAPAN2	+++	-
Panc1	+++	+
Su86.86	+++	-
Px4	+++	-
Px26	+++	-
Px117	++	-

Table 1 continued. NGN and DCC expression in tumor xenografts and cell lines[1]

Xenograft/Cell Line	*NGN*	*DCC*
Cervical		
HeLa	+++	+
HT3	+++	-
SiHA	+	-
Caski	+++	-
C4II	++	+/-
C33A	+++	+

Legend

[1]Expression studied in the majority of samples by RNase protection, as well as by RT-PCR assays with two sets of *NGN* primers and two set of *DCC* primers. The relative levels of NGN and DCC expression in the tumor specimens were designated using the following scoring system: (-) no expression detected by either RNase protection and/or RT-PCR studies; (+/-) no detectable expression by RNase protection, but faint RT-PCR signals detected; (+) low level expression detected by RNase protection and/or RT-PCR; (++) moderate expression detected by RNase protection and/or RT-PCR; (+++) high level expression detected by RNase protection and/or RT-PCR.

[2]The AsPC1 cell line has a *DPC4* missense mutation. The three other pancreatic cell lines and the three tumor xenografts (Px4, Px26, Px117) lack *DPC4* mutations.

FIGURE LEGENDS

Figure1. The predicted 1461 amino acid sequence (in single letter code) of NGN. The eight cysteines (C) in the four immunoglobulin like domains are marked by circles, and the conserved tryptophan (W) and tyrosine (Y) residues in the six FN type III domains are boxed. The eight potential N-linked glycosylation sites in the extracellular domain are indicated by solid arrows; the presumed membrane-spanning region is underlined; and the 14 potential phosphorylation sites in the cytoplasmic domain are indicated by open arrows. The sequences in the NGN cytoplasmic domain, absent in the alternatively spliced form, are boxed.

Figure 2. Western blot detection of NGN proteins. Cos-1 cells were transfected with mammalian expression vectors containing cDNAs encoding either the long (lane 1) or short (lane 2) NGN isoforms, each tagged with a VSV-G epitope. Both NGN proteins appeared to migrate at roughly 190-200 kDa, with the shorter NGN isoform migrating slightly faster. The relative mobility of pre-stained marker proteins is indicated at the left (in kDa).

Figure 3. NGN maps to chromosome 15q22. (A) The hybridization of a *NGN* P1 clone (arrow) and a chromosome 15 centromere probe (arrowhead) to human metaphase chromosomes is shown. (B) The regional assignment of NGN to 15q22 was based on its position relative to chromosome 15 landmarks.

Figure 4. Northern blot analysis of *NGN* and *DCC* expression. Northern blots containing approximately 2 μg of Poly(A+) RNA in each lane were hybridized to *NGN* (panel A) or *DCC* (panel B) cDNA probes. Following hybridization to *NGN* or *DCC*, the blots were stripped and rehybridized with a β-actin cDNA probe. The lanes contain RNA from heart (Hrt), brain (Brn), placenta (Pla), lung (Lng), liver (Liv), skeletal muscle (Skm), kidney (Kid), pancreas (Pan), spleen (Spl), thymus (Thy), prostate (Pro), testis (Tst), ovary (Ova), small intestine (S Int), colon

(Col), and peripheral blood cells (P Bld). The mobility (in kb) of molecular weight markers is indicated at the right.

Figure 5. Ribonuclease (RNase) protection assay of *NGN* expression in cancer. Samples were glioblastoma xenografts (lanes 1-10) and colorectal (lanes 11-14), breast (lanes 15 and 16), and neuroblastoma (lanes 17-20) cancer cell lines. Ten μg of RNA from a negative control rat cell line, Rat1, was loaded in lane 21. Approximately 500 cpm of the undigested *NGN* and γ-actin riboprobes were loaded in lanes 22 and 23, respectively. The relative mobility of the protected *NGN* and γ-actin fragments is indicated. The specific xenografts and cell lines were: lane 1 - 397; lane 2 -398; lane 3 -408; lane 4 - 409; lane 5 - 425; lane 6 - 443; lane 7 - 493; lane 8 - 542; lane 9 - 561; lane 10 - 566; lane 11 - DLD1; lane 12 - LoVo; lane 13 - WIDR; lane 14 -Hct 116; lane 15 - SKBR3; lane 16 - MDA-MB-361; lane 17 - SJNB-14; lane 18 - SJNB-17; lane 19 - SJNB-20; lane 20 - IMR32.

Figure 6. RT-PCR assay of *NGN* and *DCC* expression in cancer. Shown are Southern blots of the RT-PCR products generated with *NGN* cytoplasmic domain and *DCC* extracellular domain primers, and ethidium bromide-stained β-actin RT-PCR products. Pancreatic cancer cell lines and xenografts in lanes 5-7, respectively; cervical cancers in 8-13; breast cancers in 14 and 15; colorectal cancers in 16-18; neuroblastomas in 19 and 20; glioblastomas in 21-26; and control samples in lanes 27-30. The specific lines were: lane 1 - AsPC1; lane 2 - CAPAN2; lane 3 - Panc 1; lane 4 - Su86.86; lane 5 - Px4; lane 6 - Px26, lane 7 -Px117; lane 8 - HeLa; lane 9 - HT3; lane 10 - SiHA; lane 11 -Caski; lane 12 - C4II; lane 13 - C33A; lane 14 - MDA-MB-231; lane 15, MDA-MB-361; lane 16 - LoVo; lane 17 - Hct116; lane 18 - DLD1; lane 19 - SJNB7; lane 20 - IMR32; lane 21 - GBM 54; lane 22 - GBM 398; lane 23 - GBM 408; lane 24 - GBM 640; lane 25 - GBM 317; lane 26 - GBM 270; lane 27 - no RNA (negative control for RT and PCR); lane 28 - liver cDNA library; lane 29 - fetal brain cDNA library; lane 30 - no cDNA (control for PCR). The exposure times of all *NGN* lanes was 2 hr, and the exposure time of all *DCC* lanes was 6 hr.

Figure 1

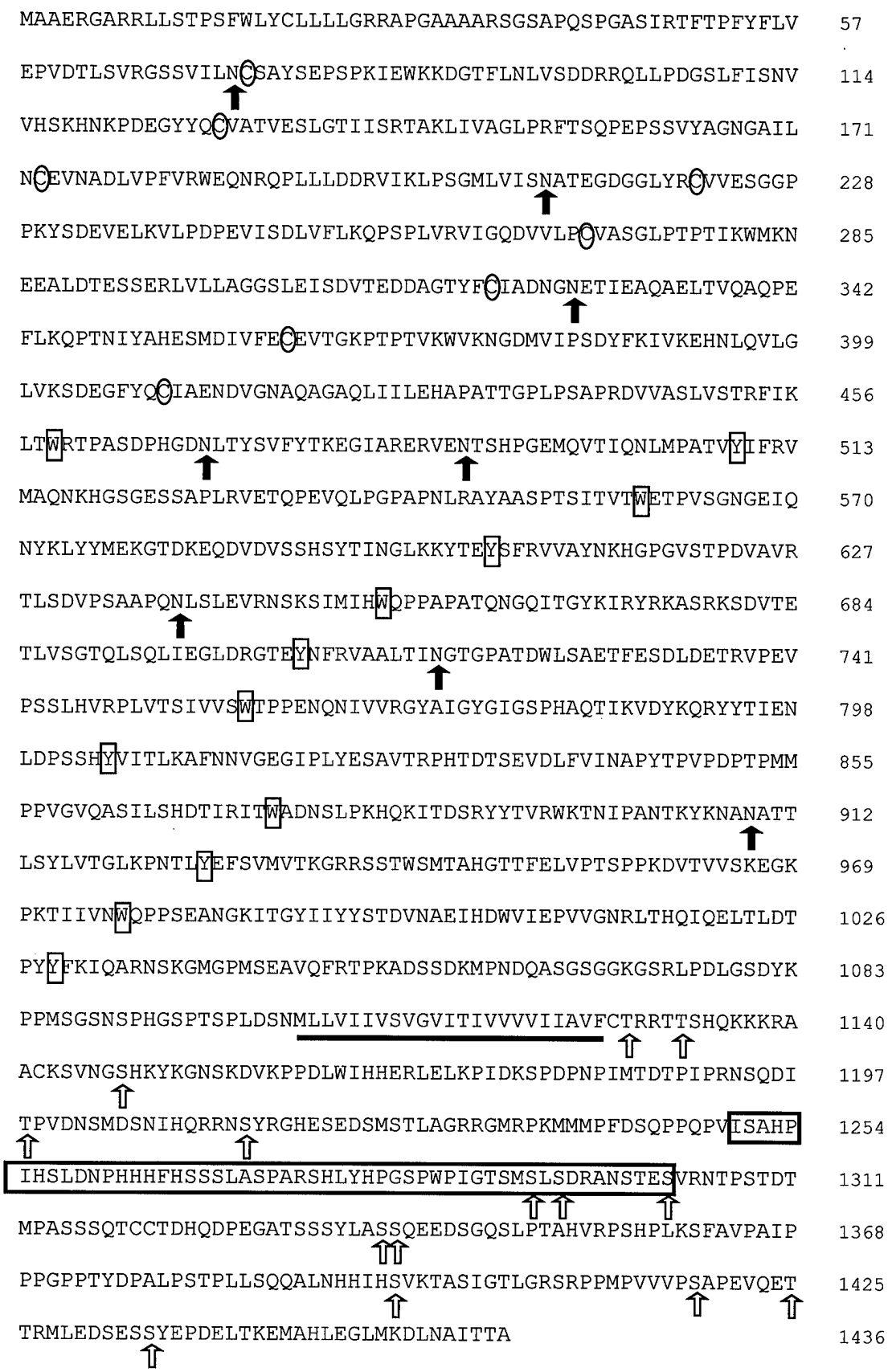
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Figure 2

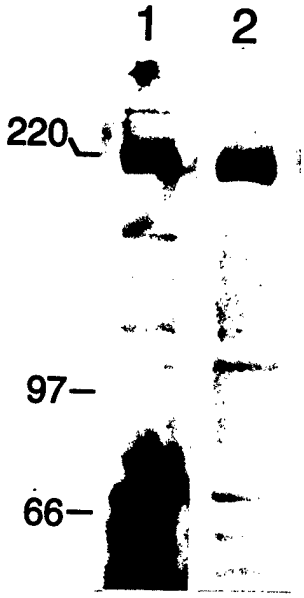


Figure 3a

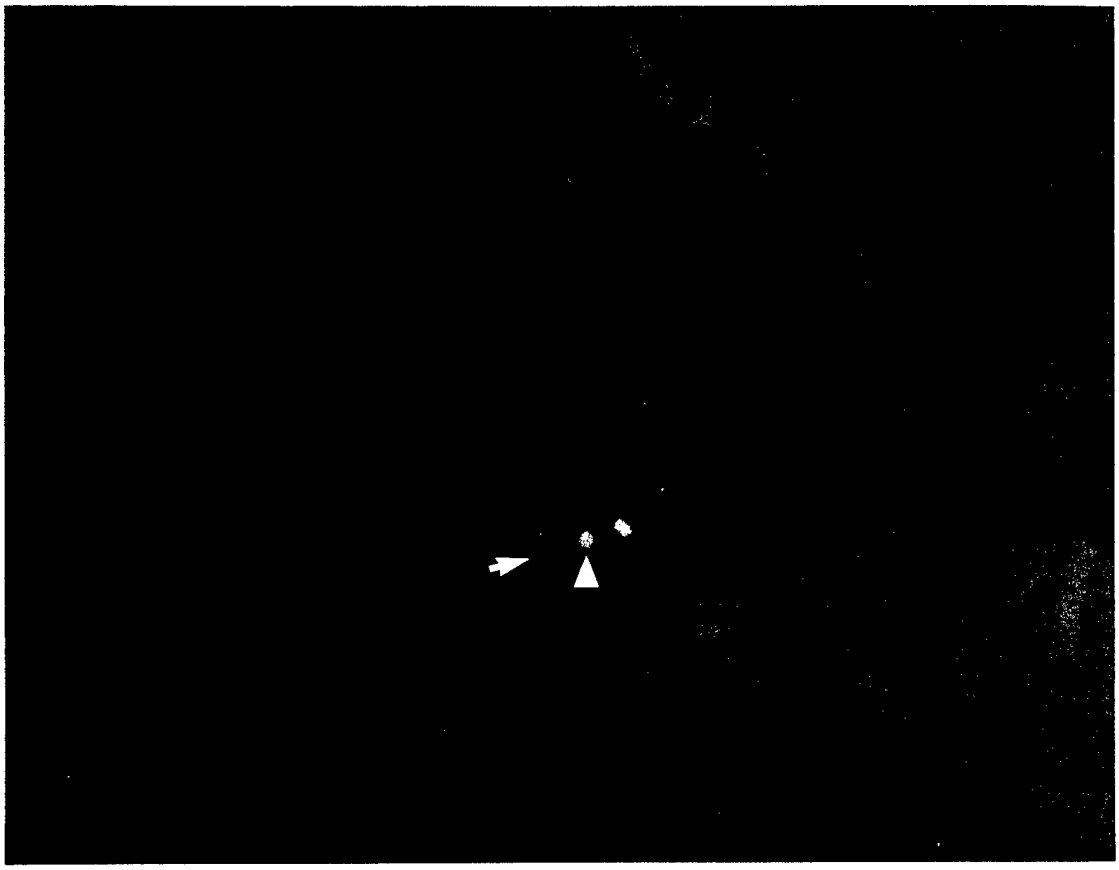
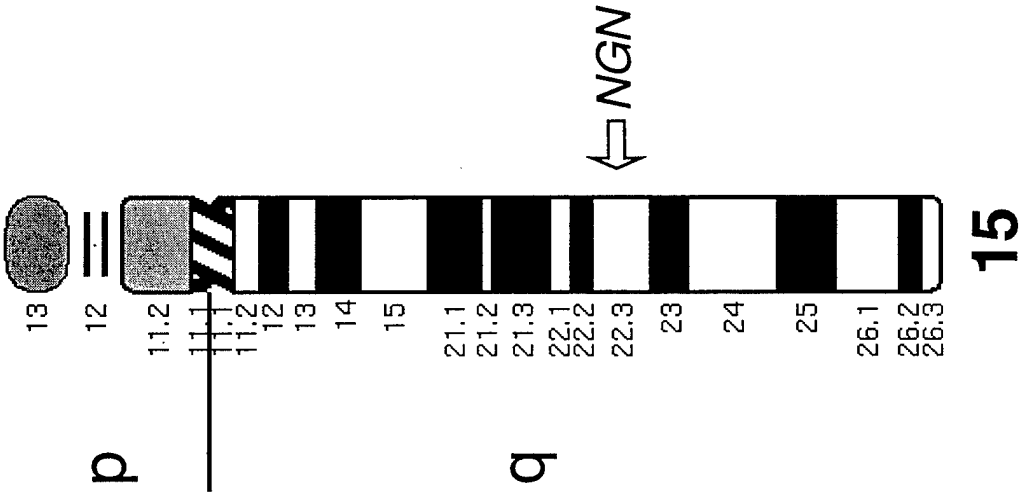


Figure 3b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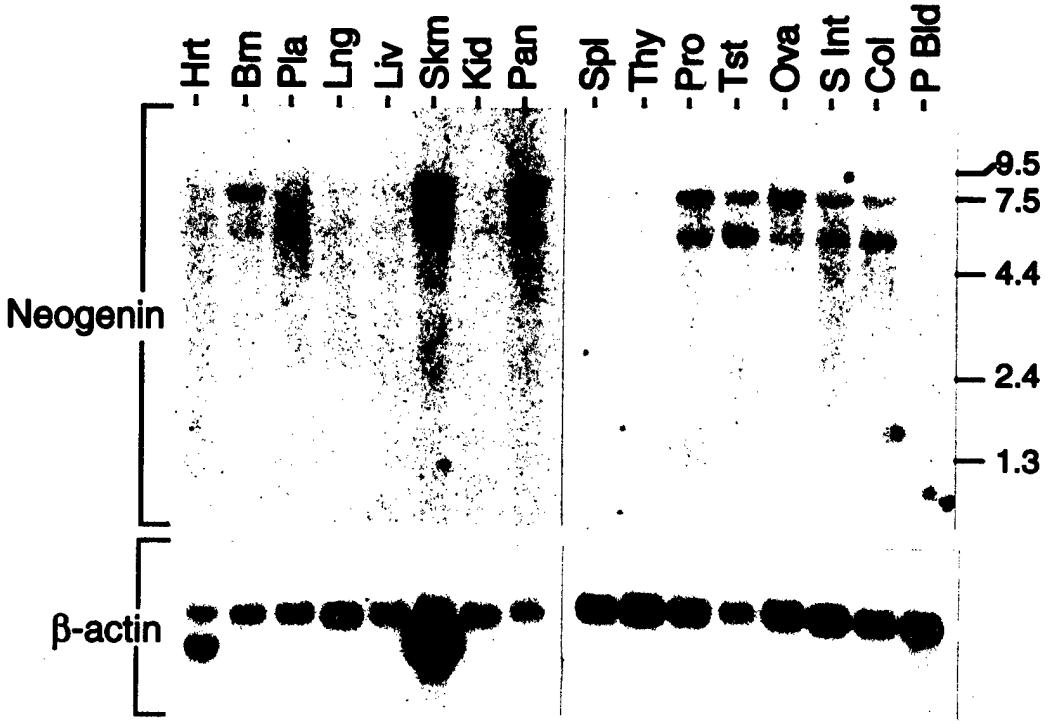
15
p
q
13
12
11.2
11.1
11.1
11.2
12
13
14
15
21.1
21.2
21.3
22.1
22.2
22.3
23
24
25
26.1
26.2
26.3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-Kid
-Pan
-Spl
-Thy
-Pro
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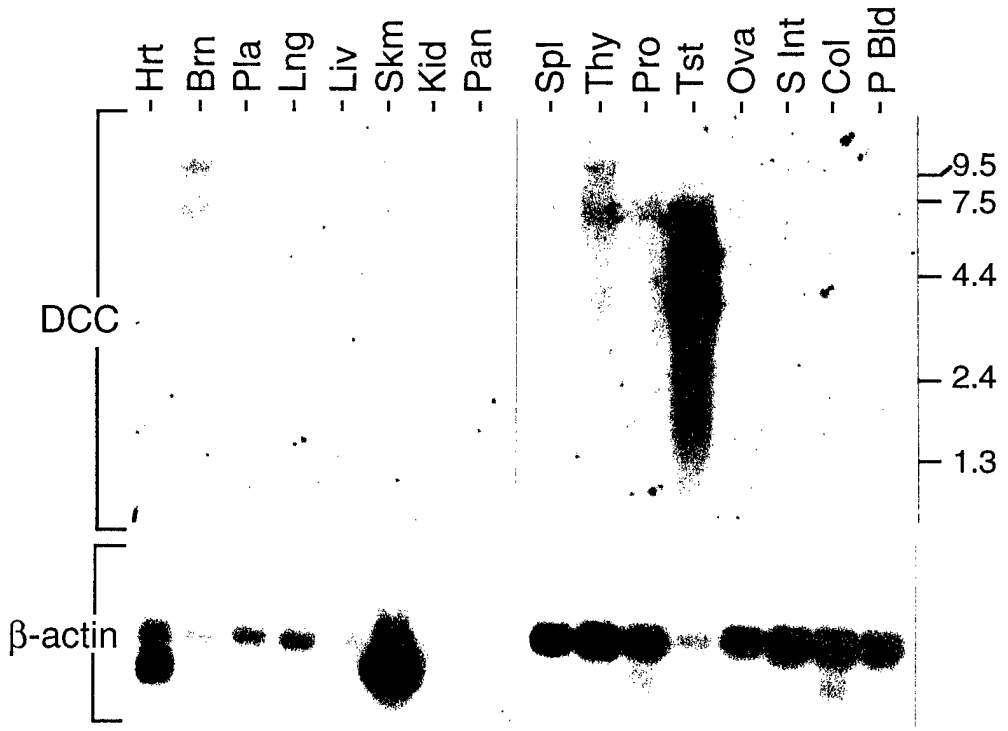
-S Int
-Col
-P Bld
9.5
7.5
4.4
2.4
1.3
Neogenin
β-actin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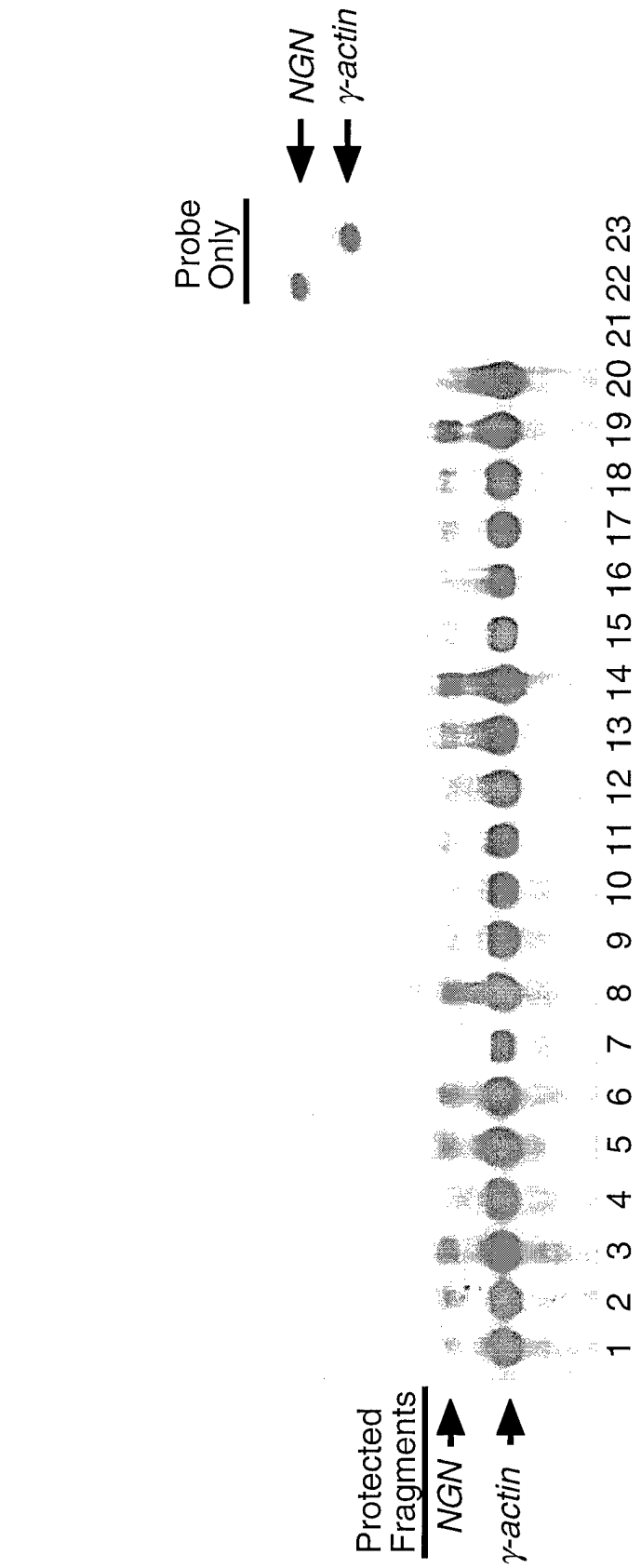
2.4
1.3
DCC
β-actin

Figure 5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γ-actin
Protected Fragments
NGN
γ-actin
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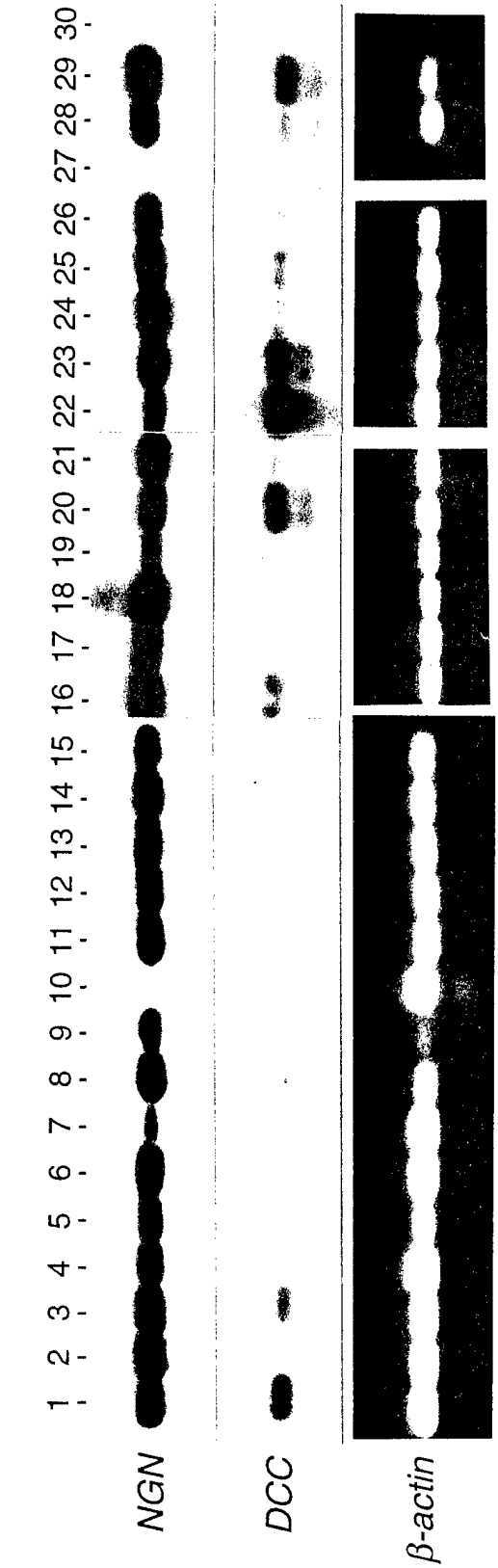
Figure 6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NGN
DCC
β-actin